Sex allocation and gene flow in Ranunculus bulbosus L

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SEX ALLOCATION AND GENE FLOW

IN

RANUNCULUS BULBOSUS L.

submitted by ADRIENNE J. STAMP
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ABSTRACT

In a study of a population of *Ranunculus bulbosus L.* at Rainbow Wood near Bath, Avon, a wide variety of gamete ratios was exhibited. The source of this variation was investigated in a series of experiments in which measures of allocation to male and female gametes were obtained. Plants were cloned and subjected to a variety of stresses in order to determine whether the gender preference of an individual could be altered by environmental factors. Relative allocation was found to vary through time and between clones. In one experiment, allocation strategy was found to vary between plants grown in sand and those grown in peat. The effect was not consistent across clones.

Levels of gene flow were also determined using estimates of pollen and seed dispersal distances and variances. Pollen dispersal was determined using measurements of bee flight distances, together with estimates of pollen carryover. Both pollen and seed dispersal were shown to be restricted. Pollen travelled further than seed (mean distances were 1.15 m for pollen and 0.027 m for seed). Pollen dispersal showed a leptokurtic distribution pattern.

Estimates of pollen and seed dispersal variances were used to calculate neighbourhood size and area. *R. bulbosus* was found to be self-sterile. Depending upon whether or not pollen carryover was taken into account, estimates of neighbourhood size varied between 44.56 and 304.82, and estimates of neighbourhood area varied between 4.16 m^2 and 11.21 m^2. The conclusion that can be drawn from this is that this population of *R. bulbosus* experiences genetic drift, but at moderate levels, and that this will have a rôle to play in determining the genetic structure of the population.

CHAPTER 1

GENERAL INTRODUCTION

The aim of the research presented in this thesis has been to understand, through investigation, aspects of gender selection and gene flow in *Ranunculus bulbosus L.* Particular attention has been paid to sexual allocation within and between individuals and to gene flow within populations. By way of an introduction, this chapter consists of a brief account of the taxonomy, morphology, ecology and reproduction of the species. Discussion of the theory of sex allocation and its application in hermaphroditic organisms, together with a discussion of gene flow, will be postponed to Chapters 3 and 4 respectively.

1.1 TAXONOMY AND MORPHOLOGY

Members of the family *Ranunculaceae* are divided into 48 genera, 23 of which are found in Europe (Tutin, *et al*, 1964). They are herbs or, rarely, woody climbers, with leaves alternate and exstipulate, rarely opposite or stipulate. Flowers are usually hermaphrodite, actinomorphic and hypogynous. The perianth is petaloid or sepaloid and whorled. Honey leaves (petaloid structures bearing nectaries) are often present and are funnel

shaped or petaloid. Stamens are numerous, usually spirally arranged and extrose. Carpels vary in number from one to many and are usually free and spirally arranged. Fruit is usually of one or more follicles or a head of achenes.

There are 131 species belonging to the genus *Ranunculus L.* in Europe, 22 of which are recognised as occurring in Great Britain by Clapham *et al*, 1962. Members of the genus are annual or perennial herbs, sometimes aquatic, with flowers solitary or in cymose panicles. They have 3 - 7 perianth segments (sepals) and 0 - 12 honey-leaves which are petaloid, yellow, white or reddish. Achenes are numerous, usually with a persistent glabrous style. Leaves are simple or palmately divided (Tutin, *et al*, 1964). Of the 23 species found in Great Britain *Ranunculus acris L., Ranunculus repens L., and Ranunculus bulbosus L.* are the most common.

R. bulbosus is tentatively divided into six subspecies: *R. bulbosus* subsp *bulbosus, castellanus, bulbifer, aleae, adscendens* and *gallecius* (Tutin, *et al*, 1964). It is a perennial herb 15 - 40 cm tall. Its stems are erect or ascending, hairy, with spreading hairs below and appressed hairs above (Tutin, *et al*, 1964). It arises from a corm-like stem tuber 0.6 - 1.15 (- 4.5) cm, which is rounded or flattened, with enlarged leaf bases above and fleshy contractile roots below. Corm size varies depending upon conditions. In the absence of competition corms can reach up to 3 cm diameter, but on permanent

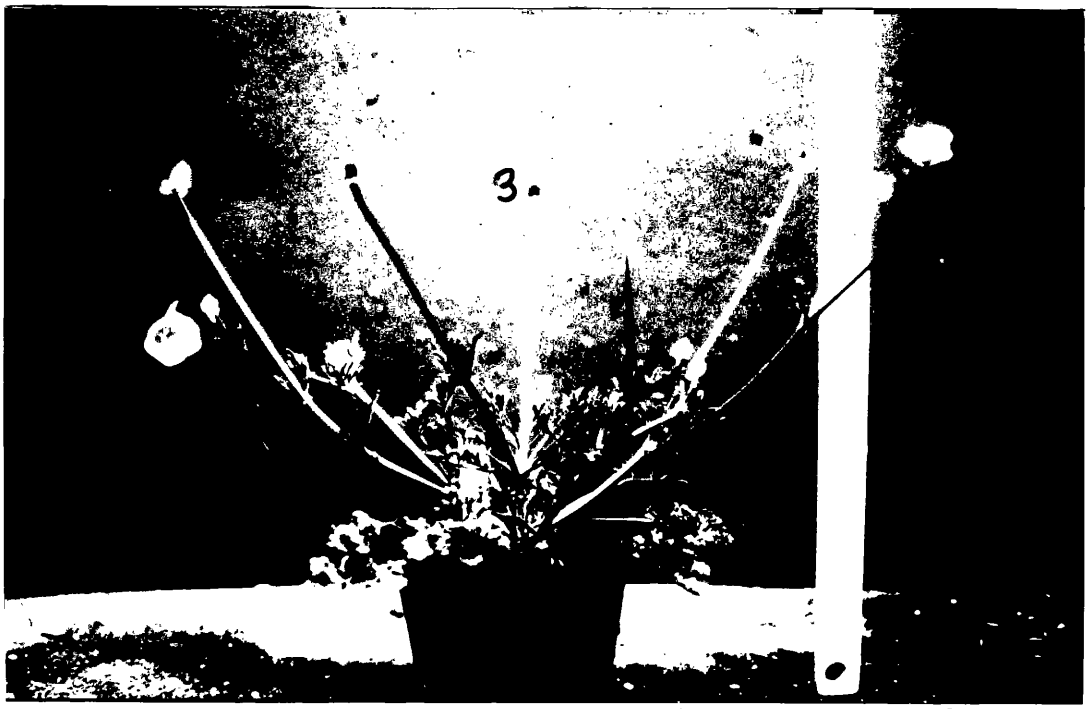


Fig 1.1 A R.bulbosus plant exhibiting flowers at all stages (1 - unopened bud, 2 - open flower, 3 - ripening seeds)

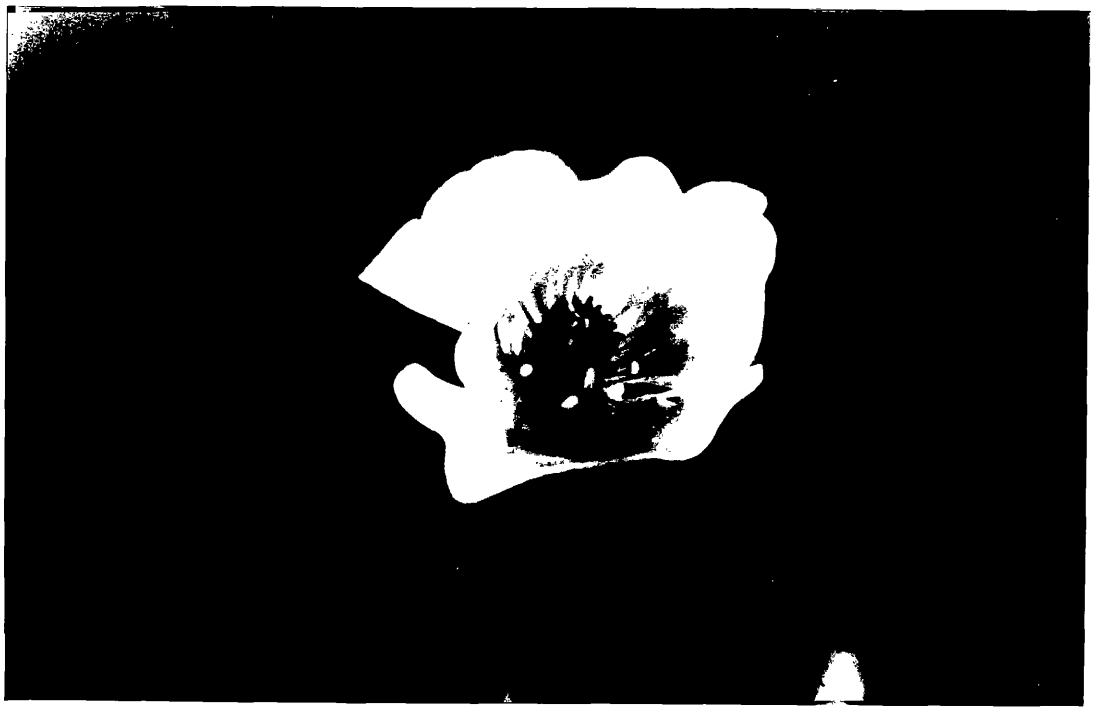


Fig 1.2 A fully open R.bulbosus flower exhibiting numerous anthers and carpels

grassland they are seldom more than 1.5 cm diameter (Harper, 1957). The basal and lower stem leaves are stalked, ovate in outline and three-lobed, the middle lobe being long-stalked. Upper stem leaves are sessile and deeply cut into narrow, often linear, segments. All leaves are usually hairy. Flowers (see Figs. 1.1 and 1.2) are 1.5 - 3.0 cm in diameter and are grouped in irregular and often corymbose cymes or are (rar=ly) solitary. Their stalks are hairy and are furrowed. Each flower has five pale, yellowish sepals which are hairy beneath and strongly reflexed, and five broadly obovate, cuneate, bright yellow (rarely paler or white) petals, which are 6 - 20 mm long. The receptacle is hairy. Flowering occurs from March to June. Most plants produce a single flowering stem but plants with large corms can produce up to eight. The flowers are protogynous and often self-sterile, although the literature is contradictory on this last point (cf Coles, 1973; Fryxell, 1957; Marsden-Jones and Turrill, 1929 a, 1952).

R. bulbosus seeds are approximately 3 mm in diameter, obovate with their faces finely pitted. They are dark brown with a paler border and possess a short, somewhat hooked, beak.

1.2 ECOLOGY

R. bulbosus is native throughout most of the British Isles (although not in Shetland) and throughout Europe to 60 °N and 24 - 28 °E. It is absent from extreme Southern Italy, most of the

Balkan peninsula, and occurs locally in Morocco, Tunisia, Georgia and Azerbaijan. It has been introduced into America and New Zealand (Tutin, *et al*, 1964).

Locally, its distribution is affected by drainage and land management. It is abundant on dry pastures, grassy slopes and fixed dunes. However, its relation to drainage pattern is best seen in a 'ridge and furrow' situation where (in a well-drained field) *R. bulbosus* will be found on top of ridges, *R. repens* in the furrows, and *R. acris* in intermediate zones (Harper, 1957). So, *R. bulbosus* grows best on well-drained soils and is particularly abundant where large areas fulfilling this condition are found, e.g. Oolitic limestone and chalk formations. *R. bulbosus* is not, however, a calcicole, and is not exclusive to such alkaline soils (Barling, 1955; Harper, 1957).

The greatest densities of *R. bulbosus* are often found on areas of intensive grazing. *R. bulbosus* contains up to 1% w/w ranunculin, so the plants are often deliberately avoided by grazing animals. Ranunculin is a glycoside which releases the toxic vesicant protoanemonin when plant tissues are damaged, e.g. during grazing (Harper, 1983). The lack of defoliation, resulting in almost uninterrupted growth, gives the plant a competitive advantage. Entry of *R. bulbosus* is made easier by overgrazing, for seedling establishment is normally observed in only small patches bared by close grazing or on newly exposed soil (e.g. mole hills). This may result in the marked clumping of

plants and seedlings seen in many grassland communities (Harper, 1957).

The ploughing and cropping of land causes a rapid reduction in population density (Barling, 1955; Chancellor, 1985) as does trampling (e.g. where footpaths cross fields giving buttercup-free 'lanes' conspicuous in the flowering season - Harper, 1957). *R. bulbosus* cannot compete with taller growing herbage due to the effect of shading (Harper, 1957). Competition is generally avoided by an early growth and reproductive cycle before other plants become too tall. Further competitive advantage is conferred as a result of the strong epinastic response of the lower leaves of the rosette which enable the plant to form a saucer shaped hollow in the turf from which other species are excluded (Harper, 1957).

R. bulbosus passes the midsummer period in a dormant state with no above-ground parts. In the Autumn, a lateral bud of a corm becomes active and develops by the growth of an intercalary meristem into a lateral bud which forms a rosette of leaves. The rosette stays green over the Winter. The base of the new shoot swells to form a new corm which reaches its mature size in the Spring. In February and March new leaves are added to the rosette, at which time starch reserves from the old corm disappear. Starch reserves are laid down in the crown of the new shoot. After the new shoot has grown, flowering occurs (May - June) by which time the old corm has usually decayed.

Immediately after seed-set, the above-ground parts of the plant die. Re-activation of the plant may occasionally occur, if rainfall is plentiful, at the end of July or in August (Harper, 1957). In this case a lateral bud is initiated from an axil of the corm. It develops into a new shoot at the base of which a new corm is formed. Harper (1957) reports that two cycles of flowering and corm formation can be induced when plants are maintained in waterlogged conditions. He suggests that the fact that each cycle involves new corm formation and the death of all above-ground parts shows that the onset of dormancy is an inherent sequel to flowering, but that breaking dormancy requires only "adequate moisture and temperature suitable for growth".

Sarukhán (1976) studied mortality curves in *R. bulbosus*, *R. acris* and *R. repens*. He found that the main periods of mortality tie in with certain events in the life cycles of the three species. All three showed mortality peaks during Spring to early Summer, with *R. bulbosus* having the earliest peak. It is during the spring and summer that the three species grow and reproduce. All three showed pre- and post-reproductive peaks of mortality, together with a trough during maximum flowering. The timing of these events was reflected in the order in which the three species enter their reproductive phases. *R. bulbosus* begins its reproductive cycle first, *R. repens* last. Newly emerged seedlings of *R. bulbosus* and *R. acris* showed high mortality rates. *R. repens* reproduces mainly through vegetative means and so this peak was not apparent. Seedling mortality in *R. bulbosus* occurs

at three stages: a) pre-emergence in the Autumn b) post-emergence due to damping off (particularly of etiolated seedlings found in long grass) c) in dry periods when corm development is insufficient to sustain the seedling (Harper, 1957).

Life cycle events also reflect themselves in the peak period of biomass production. Sarukhán (1976) reports three phases in the biomass production curves : rapid growth of Winter rosettes from April to May/June; followed by a period of almost zero growth at the time of reproduction (vegetative or sexual); and finally a period of vegetative growth after reproduction. Sarukhán also studied how biomass is divided between different structures in the three species. *R. bulbosus* exhibited differences from the other two species in that a relatively small proportion of the biomass was found in the roots with a relatively high proportion in the corm. Sarukhán reports that this suggests Summer dormancy may permit it to avoid water stress. He also suggests that the food storage rôle of the corm in *R. bulbosus* is filled by the roots in *R. repens* and *R. acris*.

1.3 REPRODUCTION

R. bulbosus reproduces sexually. It may also reproduce vegetatively, by splitting of the corm, although there is some dispute as to the extent this contributes to overall reproduction. Barling (1955) reports that vegetative reproduction, through the production of more than one shoot from a single corm, is a common

feature of Autumn growth. He does not, however, say if each shoot then forms its own new corm which would result in the formation of completely separate new plants (ramets). Harper (1957) and Coles (1973) suggest that reproduction by vegetative means occurs only occasionally, but may be more common under favourable conditions. Sarukhán and Gadgil (1974) report that *R. bulbosus* reproduces exclusively by seed production but Sarukhán, in a paper from the same year (Sarukhán, 1974), states that it depends almost entirely on seed production for reproduction.

R. bulbosus seeds have a half-life of approximately eight months, so that with constancy of seed production there is little overlap between seed populations from one year to the next (Sarukhán, 1974). Sarukhán (1974) carried out a study of seed population dynamics in *R. repens, R. acris* and *R. bulbosus*. He found that in the first year of study, *R. acris* and *R. bulbosus* produced similar numbers of seeds per m^2 where density of plants was constant ($800/m^2$ and $982.5/m^2$ respectively). *R. acris* and *R. bulbosus* produced six times as much seed as *R. repens* (which produced $149.5/m^2$). In the second year of study *R. bulbosus* produced ten times as much seed as both *R. acris* and *R. repens* ($162.9/m^2$ compared with $15.6/m^2$ and $16.6/m^2$ respectively). Seeds were lost from the population by predation by cows and small mammals such as voles.

Harper (1957) recommends conditions for germination of seeds. He states that seeds sown directly after harvesting from the field (when they can be shaken off the receptacle) give poor

germination but that successive sowings give increased germination until twelve weeks after harvesting, when the seed reaches maximum viability (96%). This suggests that there is an initial period of dormancy or after-ripening. If seeds are exposed to damp conditions before dormancy is complete they lose viability. Harper also suggests that treatment for ten days at 0 °C reduces percentage germination. He obtained highest germination rates in light, sandy soil, with seeds sown at a depth of approximately 1.6 cm.

Sarukhán (1976) reports that *R. bulbosus*, together with *R. acris*, have high reproductive efficiencies when compared with *R. repens* (15%, 11% and 1 - 5% respectively). However, if both seed and vegetative reproduction are taken into account, the 3 species are very similar, their total allocation to reproduction being respectively 49 - 52%, 48 - 60% and 48 - 50%. These final figures take into account allocation to achenes and ancillary structures and allocation to vegetative reproduction for the period June 23 - July 16 1969.

R. bulbosus plants, although reported to be hermaphrodite (Tutin, *et al*, 1964) exhibit a whole range of gender states from completely hermaphrodite to female (no viable pollen) or male (no functional ovules). Individuals lacking both viable pollen and ovules are occasionally found. These states have been reported in several papers (Burkhill, 1895; Clapham *et al*, 1962; Marsden-Jones and Turrill, 1929 a, b, 1952).

Although *R. bulbosus* is reported as being self-sterile (Tutin, *et al*, 1964) there has been some debate upon this matter. Marsden-Jones and Turrill (1929 a, 1952) report that both selfing and aposporous apomixis may occur. Coles (1973) and Fryxell (1957), however, report *R. bulbosus* as being self-incompatible and obligately sexual. Coles (1973) suggests that apparent self-fertilization reported by other authors resulted from accidental contamination with foreign pollen.

Sex determination in *R. bulbosus* has not been widely studied. Harper (1957) supports the general sex determination scheme suggested by Crane and Lawrence (Crane and Lawrence, 1938 - quoted from Harper,1957) for *Rubus* as a viable scheme for *R. bulbosus.* That is, M and m allelomorphs for maleness and F and f allelomorphs for femaleness. McArthur (1977) proposed a sex-determining mechanism which accommodates the varying gender ratios he found in *Atriplex canescens.* He investigated a tetraploid, half-sib family of 655 individuals, which he evaluated annually for floral phenotype. He found that 61 plants changed sex in more than one year, and 107 changed in only one year. He speculated about a sex-determining mechanism in which the plant has at least four chromosomes with at least one locus involved in sex determination. He assumed that a tetraploid zygote must have two or more X's to live, which gives three viable combinations: XXXX; XXXY; XXYY. He suggested that XXXX represents pistillate or 'female' plants, and XXYY represents staminate or 'male' plants (neither of which can vary their gender), and XXXY could be

pistillate or staminate, or both, and could vary gender depending upon environmental conditions. He supports the theory with the observations he made on his experimental population. *R. bulbosus* is not, however, tetraploid, but has been reported as exhibiting varying sex ratios (see earlier for references).

CHAPTER 2

GENERAL METHODS

2.1 INTRODUCTION

This chapter includes general information on the collection and propagation of the plants used during this research. Methods specific to individual experiments are given in the appropriate chapters.

2.2 EXPERIMENTAL PLANTS

All plants used in these experiments were members of *R. bulbosus*. Experimental plants were originally collected from three sites close to the University of Bath, Avon:

i) Sham Castle Meadow (SCM) - an area of cattle grazed permanent pasture 1 km to the east of the university.

ii) Rock Border (RB) - a rocky border, covered by shallow soil along the edge of a road leading into the university from the east.

iii) Firing Range (FR) - an area of permanent grassland (rarely cut and not grazed) on the university's firing range on the west side of the university campus.

After plants had been collected they were potted in Fisons' M2 compost in 4.5" pots and left outside until required.

All field studies were carried out on National Trust land in the field below Rainbow Wood (grid reference ST 76 71). This field is permanent pasture, grazed only by a small herd of Welsh Black Cattle and by wild rabbits. A list of some of the other species growing in the field at Rainbow Wood is given in Tables 2.1a and 2.1b.

The area is inhabited by *Lasius flavus* ants which build mounds in the field. These mounds grow bigger every year and estimates of their age can be obtained by measuring their size (Franks, N.R. pers. comm.). From these estimates it is known that the area has not been cultivated for at least 100 years.

2.3 VEGETATIVE PROPAGATION OF *R. BULBOSUS*

In order to have access to several genetically identical individuals, investigations were undertaken to determine a reliable method of vegetatively propagating *R. bulbosus*. Four methods were investigated:

TABLE 2.1a

OTHER NON-GRASS SPECIES FOUND AT RAINBOW WOOD

NON-GRASS SPECIES	
SCIENTIFIC NAME	COMMON NAME
Achillea millefolium	Yarrow
Ajuga reptans	Bugle
Bellis perennis	Daisy
Cardamine pratensis	Lady's-smock
Cerastium fontanum	Common Mouse-ear
Cirsium eriphorum	Woolly Thistle
Conopodium majus	Pignut
Crepis spp.	Hawksbeard
Dactylorhiza fuchsii	Common Spotted Orchid
Hypochoeris radicata	Common Cat's-ear
Leontodon hispidus	Rough Hawksbit
Leucanthemum vulgare	Oxeye Daisy
Linum carthariticum	Fairy Flax
Lotus corniculatus	Common Bird's-foot-trefoil
Medicago lupulina	Black Medick
Myosotis arvensis	Field Forget-me-not
Pilosella officinarum	Mouse-ear Hawkbit
Plantago lanceolata	Ribwort Plantain
Plantago media	Hoary Plantain
Primula veris	Cowslip
Ranunculus acris	Meadow Buttercup
Ranunculus repens	Creeping Buttercup
Rumex acetosa	Common Sorrell
Sanguisorba minor	Salad Burnet
Senecio vulgaris	Groundsel
Silene spp.	Campion or Catchfly
Taraxacum spp.	Dandelions
Trifolium pratense	Red Clover
Trifolium repens	White Clover
Veronica chamaedrys	Germander Speedwell

TABLE 2.1b

GRASS SPECIES FOUND AT RAINBOW WOOD

GRASSES	
SCIENTIFIC NAME	COMMON NAME
Agropyron repens	Couch Grass
Anthroxanthum odoratum	Sweet Vernal-grass
Arrhenatherum elatius	False Oat Grass
Briza media	Common Quaking Grass
Bromus erectus	Upright Brome
Cyanosurus cristatus	Crested Dog's-tail
Dactylis glomerata	Cocksfoot
Poa annua	Annual Meadow-grass
Poa pratensis	Smooth Meadow-grass

i) SPLITTING THE CORM

1. A whole plant was removed from its pot and excess soil was removed from the root system.
2. The roots were cut away from the corm and put to one side, leaving the corm and its associated rosette of leaves intact. This was cut into quarters.
3. The quarters were cut into pieces approximately 1 cm^2 with at least two firmly attached leaves. Any loose leaves were removed.
4. The cuttings were planted in a mixture of peat, sand and perlite (ratio 3:2:1 v/v respectively) just deep enough so that they were supported but not buried. Not more than six cuttings were planted in a 4.5" pot.
5. The cuttings were drenched with Benylate fungicide solution at a concentration of 1 g/l, and then well watered.
6. The cuttings were left under mist in a heated greenhouse (minimum temperature 18 °C, natural daylight/length) until they had rooted.
7. Rooted cuttings were planted singly into 3.5" pots of Fison's M2 compost and left in an unheated greenhouse until established. Once established, they were placed outside until required.

ii) TAKING ROOT CUTTINGS

1. Roots set aside from plants used for method i) were used. Any roots under 1.5 mm in diameter or hollow were discarded. The remaining roots were cut into 2 cm lengths with the end furthest away from the corm being cut to a point to help with orientation in the soil.

2. The root cuttings were planted in trays of the same mix of soil as used in method i). They were orientated with the pointed end down and the top of the cutting just showing above the soil surface. Cuttings were planted at a spacing of 2 cm.

3. As from point 5 in method i).

iii) PROPAGATING SINGLE LEAVES

1. A plant was removed from its pot and excess soil removed.

2. Single leaves were cut away from the corm as near to their bases as possible.

3. Leaves were planted in pots of the same soil mix as used in methods i) and ii). No more than five leaves were planted in a 4.5" pot at a depth just sufficient to offer them support.

4. As from point 5 in method i).

iv) TISSUE CULTURE OF THE APICAL MERISTEM

1. The central portion from a plant's rosette of leaves was isolated, leaving the apical meristem, plus a small portion of non-dividing corm, intact. Any large leaves were cut away and discarded.

2. Several cuttings were stirred in a 2% hypochlorite solution for 5, 10, 15 or 20 minutes, to sterilise them, and then rinsed thoroughly with sterile distilled water.

3. The cuttings were cultured on a medium containing MS (Murashiga and Skoog) at a concentration of 4.7 g/l, to which was added lab 'm' agar mc2 (7 g/l) and sucrose (20 g/l). Then either 5 M or 1 M of BA was added, and the pH set to 5.7. The medium was autoclave sterilised (121 °C, 15 psi, 15 minutes).

4. Shoots were grown in 175 ml glass jars on 20 ml of the medium at 25 °C with irradiance of 10 w/m PAR and 16 hr daylength.

5. Shoots were assessed after four days for contamination.

The only successful method tested was propagation by splitting the corm [method i)]. Cuttings rooted after 3 - 4 weeks under mist with almost 100% success. Propagation through root cuttings and single leaves gave no success. All tissue in culture jars became contaminated. Corm splitting was, therefore, the chosen method for propagation.

CHAPTER 3

SEXUAL ALLOCATION IN *RANUNCULUS BULBOSUS L.*

3.1 INTRODUCTION

3.1.1 GENERAL CONSIDERATIONS

A number of species are known to exhibit varying allocation to male and female function, both within and between plants and populations and within and between seasons. Schaffner (1922) observed pure staminate, pure carpellate and intermediate individuals (varying from nearly pure carpellate to nearly pure staminate) in five natural populations of *Arisaema triphyllum (L)* Torr. and *Arisaema dracontium (L)* Schott. in Ohio. Barrow (1987) examined native diploid, tetraploid and hexaploid populations of *Atriplex canescens* in southern New Mexico and west Texas. He reports approximately equal male:female ratios in diploid populations and female biased ratios in tetraploid and hexaploid populations with variation in bias between populations. Bawa and Webb (1983) report that *Muntingia calabura* in a Costa Rican population produced a range of floral forms varying from flowers with a large pistil and as few as 10 stamens to flowers with a much reduced pistil and over 100 stamens. This distribution was

fairly continuous. Other studies which show variation in male and female function include Freeman *et al* (1981) on *Sarcobatus vermiculatus, Quercus gambelii* and *Juniperus osteosperma*; Freeman and McArthur (1984) on six *Atriplex* species; Lovett-Doust and Cavers (1982) on *Arisaema triphyllum* (Araceae); Richards (1988) on *Ilex aquifolium* L. and *Rhodiola rosea L.; Solomon (1985) on Solanum carolinense;* Stephenson (1984) on *Lotus corniculatus;* Thomson and Barrett (1981) on *Aralia hispida.* See Charnov (1982), Goldman and Willson (1986), Lloyd and Bawa (1984) and Willson (1979) for further references. Schlessman (1986) discusses two reports of sex changes in *Acer grandidentatum* and *Acer pensylvaricum.* In these reports, ambisexuals are viewed as being either male or female, depending upon the bias of allocation to either sex function. He suggests that the reported sex changes are therefore invalid. However, the data do appear to show gender variation if not actual sex change.

Most higher plants are fairly immobile as adults and therefore have to breed at or very close to the site of germination (Charnov, 1982). As a consequence, many plants are very plastic in their growth (Bradshaw, 1965, 1974; Lloyd, 1984 a). As both environmental factors and plant size may affect opportunities to reproduce through male or female function, it may be hypothesized that individuals will adopt the reproductive strategy most appropriate in a particular environment or at a particular size. Certainly, it has been predicted that stress should induce maleness because of the relative costs of

being male or female (Bull, 1981; Burkhill, 1985; Charnov, 1982; Charnov and Bull, 1977; Freeman *et al*, 1980; Meagher, 1981; Solomon, 1985). Since half of a zygotes' genes are inherited from the mother and half from the father, male and female function can be considered as equivalent means to reproductive success (Charnov, 1982). However, the costs of being either male or female may vary considerably. If so, it is likely that reproduction through the female function is more costly than through the male function (Charnov, 1982; Freeman *et al*, 1980; Lovett-Doust and Cavers, 1982; Willson, 1979). Indeed, Lovett-Doust and Cavers (1982) have shown that fruiting females of *Arisaema triphyllum* allocate about three times as much resource to reproduction as do males. Thus, in a resource-poor environment, a plant may increase its reproductive success by reproducing primarily, or exclusively, as a male, fertilizing the eggs of neighbours occupying more favourable environments.

A number of studies have provided evidence to support this theory. Freeman *et al* (1981) studied gender allocation in three monoecious species (*Sarcobatus vermiculatus*, the Black Greasewood; *Quercus gambelii*, the Gambel Oak, and *Juniperus osteosperma*, the Utah Juniper). They showed male function to predominate in xeric sites and female function to predominate in mesic sites. McArthur (1977) evaluated a tetraploid half-sib family of *Atriplex canescens* for floral phenotype between 1972 and 1975. The winter of 1972/3 was unusually cold and placed great stress on the plants, many of which died. Surviving plants showed a very

different sex ratio compared to the previous year. The overall numbers of males and hermaphrodites in the population increased compared to females, and 27 plants changed their sex from female to male. Solomon (1985) and Lovett-Doust and Cavers (1982) describe similar examples of changes in gender allocation in populations of *Solanum carolinense* and *Arisaema triphyllum.*

Freeman *et al* (1980) have suggested that "lability of sexual expression has survival value where a significant portion of females must otherwise bear the cost of fruit production in an unfavourable environment".

3.1.2 THEORETICAL CONSIDERATIONS

The theory of how organisms should allocate resources to the sexual functions has been called the "theory of sex allocation" (Charnov, 1982). The theory was first developed in order to predict the ratio of sons and daughters that should be produced by dioecious organisms. Fisher (1930, quoted from Charnov, 1982) showed that the stable sex ratio at conception will be 1:1 if there are no varying costs or survivorships for sons and daughters. He derived a model which introduced the idea that the relative costs and benefits of producing a son or daughter may affect the fitness of the mother through the two functions. He found that, in general, selection would favour over-production of the cheaper sex. Maynard-Smith (1980, quoted from Charnov, 1982) confirms this in his model of sex allocation. In hermaphrodites,

the decision to be made concerns the proportion of resources that should be allocated to male and female function.

The theory regarding sexual allocation in hermaphrodites is reviewed in detail by Charnov (1982). He poses two questions. When does an hermaphrodite have a higher fitness than a pure male or pure female? how does an hermaphrodite allocate resources to male and female function? Before considering these questions it is worth describing some of the earlier arguments on the advantages or disadvantages of hermaphroditism.

Hermaphroditism is a common phenomenon in the plant kingdom. The advantages of adopting an hermaphrodite life-style are many. Ghislein (1969) reviews the earlier literature on the selective advantage of hermaphroditism in animals. He identifies three major hypotheses which he described as the low density model, the size advantage model, and the gene dispersal model. The low density model considers a dioecious population in which an organism has a low probability of mating but a high probability of encountering members of the same sex. If members of this population were hermaphroditic, every individual encountered would be a potential mate. The size advantage model offers an explanation for sequential hermaphroditism. Basically, it argues that if an organism experiences age or size effects on its reproductive success through either sex, it can increase its reproductive potential by assuming the sex which is advantageous to its current age or size. The gene dispersal model argues that

hermaphroditism may be advantageous when factors limiting gene dispersal affect population structure. There are two versions of the model. The first, the inbreeding version, argues that sequential hermaphroditism may reduce the probability of inbreeding among siblings when dispersal is low. In a dioecious population, male and female siblings may mate with each other. If all members of a sequentially hermaphroditic family were synchronized in their transformation from one sex to another, there would be reduced mating among siblings. The second version predicts that small populations (caused by decreased gene dispersal) will suffer genetic drift which may result in the predominance of one sex. This would decrease the variety and number of possible crosses. If all individuals exhibited both male and female function, the number of possible crosses would increase. He provides evidence from various taxa within the animal kingdom to support these hypotheses.

Heath (1977) briefly reviews other models that ascribe advantages to hermaphroditism. Advantages may accrue whenever individuals mate rarely and males consequently produce an excess of gametes. The resources allocated to these could be more profitably allocated to female gametes. Hermaphroditism will also be advantageous if an individual is isolated from possible mates, as it will allow reproduction through self-fertilization. Finally, an hermaphrodite population may be more fecund than an equivalently sized dioecious population, as each individual of the former, but only some of the latter, exhibit the female function.

If there are so many advantages to being an hermaphrodite, why is it not a universal phenomenon? Heath (1977) considers the costs of being hermaphroditic. He argues that a dioecious individual will be able to allocate a greater proportion of its resources to gamete production than an hermaphrodite, as each individual of the latter has to produce and maintain the reproductive apparatus of both sexes. The energetic advantage to the gonochorist will depend upon the cost of producing reproductive structures. Hermaphrodites will still be favoured at population densities below a certain threshold, or when over-production of one gamete type occurs (hermaphrodites would be able to channel resources to the other sex function). Another potential cost of hermaphroditism is inbreeding depression through self-fertilization, although many hermaphrodites are self-incompatible.

Charnov *et al* (1976) argue that the breeding system (hermaphroditism or monoecy *versus* dioecy, androdioecy and gynodioecy) is determined by the shape of the fitness set. The fitness set is the trade-off between resources allocated to male and female functions. Where the fitness set is convex, hermaphroditism will be favoured. Where it is concave, dioecy will be favoured. Where it is concave-convex, androdioecy or gynodioecy will be favoured.

Charnov (1982) describes a simple model to show when an hermaphrodite will be favoured over a pure male or a pure female.

Let n_1 be the number of males in the population (each producing k_2 pollen grains), n_2 be the number of females in the population (each producing k_1 seeds), and n_3 be the number of hermaphrodites in the population (each producing $f.k_1$ seeds and $m.k_2$ pollen). m and f are the proportion of the hermaphrodites' fitness transmitted through male and female function respectively. W_f, W_m and W_h denote the fitness of the male, female, and hermaphrodite respectively (fitness being measured in terms of the number of haploid chromosome sets contributed to the offspring). K is the number of offspring produced by a population - these contain $2K$ haploid chromosome sets.

Thus:

$$W_f = \frac{K}{n_2 + fn_3} \qquad W_m = \frac{K}{n_1 + mn_3}$$

$$W_h = \frac{Km}{n_1 + mn_3} + \frac{Kf}{n_2 + fn_3}$$

If no hermaphrodites are present, $n_3 = 0$ and the Evolutionarily Stable Strategy (ESS) is a sex ratio of 0.5 (i.e. $n_1 = n_2$). An hermaphrodite can invade the population if $W_h > W_f$ or W_m, or if $m + f > 1$. A dioecious population will be stable if $m + f < 1$. The shapes of the fitness sets under these conditions are shown in Fig. 3.1.

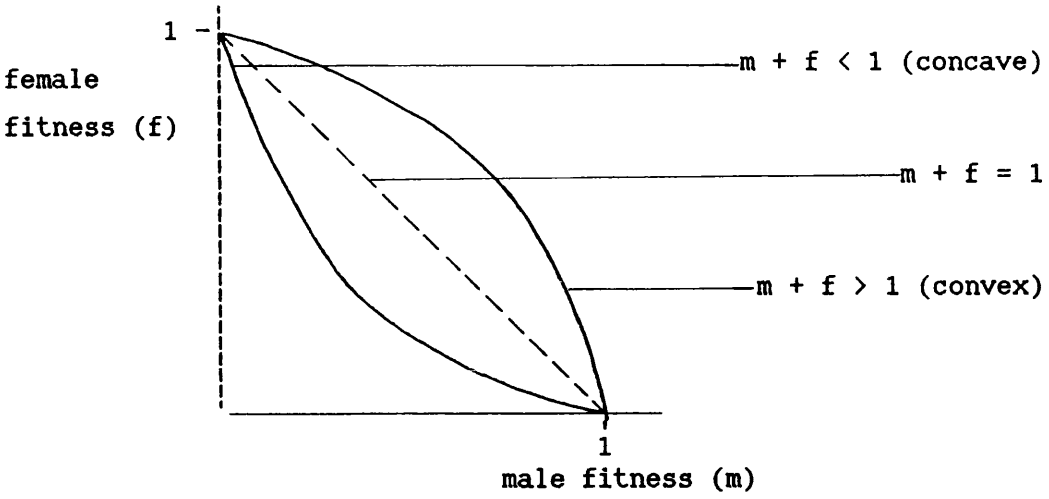


FIG. 3.1 Possible fitness sets for the trade-off between male and female reproduction in a simultaneous hermaphrodite (Charnov, 1982).

Charnov *et al* (1976) suggest three reasons why the fitness set should be convex and thus favour hermaphroditism: low mobility, low resource overlap (pollen production occurring earlier in the season than seed maturation) and cost sharing (e.g. flowers in insect-pollinated plants attracting potential fertilizers of ovules and dispersers of pollen). Goldman and Willson (1986) argue that in some cases there may be no trade-off between resources allocated to male and female functions. Whether there is a trade-off will depend upon the period of overlap of resource demand. Charlesworth and Charlesworth (1981) examine the implications for the fitnesses of male sterile and female sterile mutants when some selfing occurs. They conclude that the shape of the male *vs* female fitness set is not a good predictor of the occurrence of hermaphroditism when there is some selfing.

One of the major advantages of being an hermaphrodite is that it provides the opportunity for preferential resource allocation under varying circumstances. Charnov (1982), in a simple ESS model, investigates the fitness of a rare mutant hermaphrodite which alters its male:female fitness value. Most members of the population have proportional fitness values of $\underline{m}$ and $\underline{f}$ through male and female function respectively and overall fitness of $\underline{W_h}$. The mutant has proportional fitness values $\hat{\underline{m}}$ and $\hat{\underline{f}}$ and overall fitness of $\hat{\underline{W}}_h$. Thus:

$$\hat{W}_h = \frac{\hat{m}}{n_3 m} + \frac{\hat{f}}{n_3 f} \propto \frac{\hat{m}}{m} + \frac{\hat{f}}{f} .$$

$\underline{n_3}$ is the number of individuals with values of $\underline{m}$ and $\underline{f}$ in the population. If $\hat{\underline{W}}_h > 2$, the mutant is selected for, if $\hat{\underline{W}}_h < 2$, it is selected against. The ESS values $(\underline{m}^*, \underline{f}^*)$ are the values at which the product $\underline{m}^*.\underline{f}^*$ is maximized.

Charnov (1982) shows that $\underline{m}^*, \underline{f}^*$ may also be found by looking for a pair of $\underline{m}^*, \underline{f}^*$ such that a line through that pair with a slope $\underline{-f^*/m^*}$ is a tangent to the fitness set at the ESS values (Fig. 3.2).

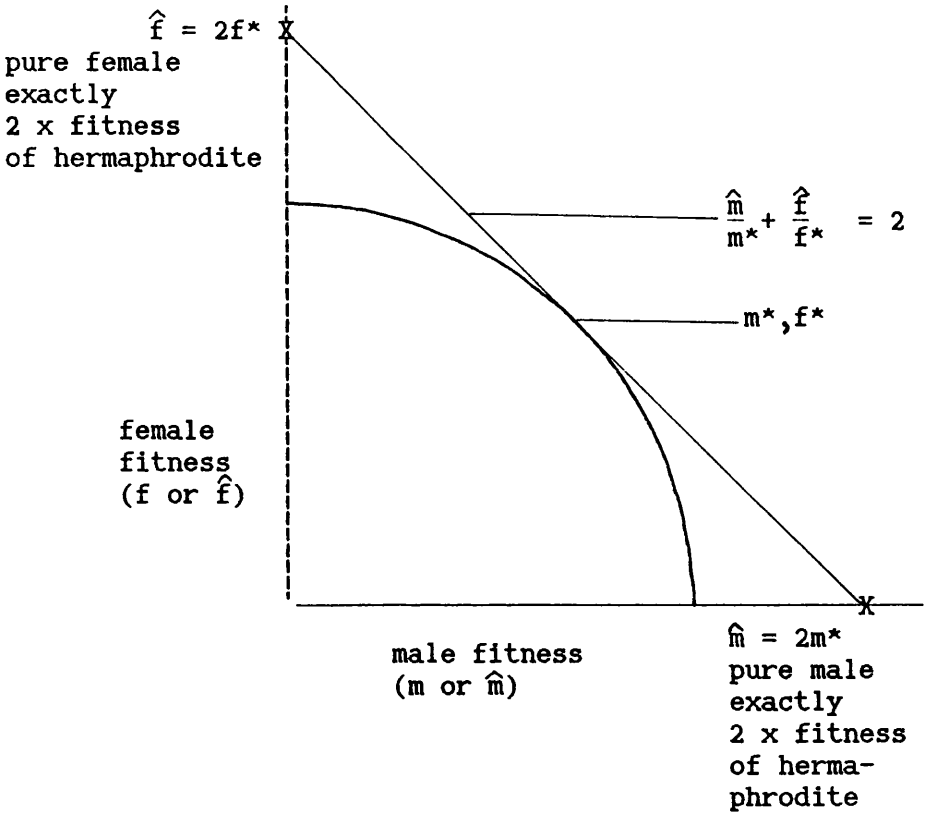


FIG. 3.2 The ESS allocation to male *versus* female function maximizes $\underline{m}^*, \underline{f}^*$ (Charnov, 1982)

This again predicts that if the fitness set is convex, hermaphroditism will always be favoured, but if it is concave, dioecy will be favoured.

A different shape of fitness set may give rise to a different $\underline{m}^*, \underline{f}^*$ value. The shape of a fitness set may vary from year to year and in different environments. What determines the shape of the fitness set?

The shape of the fitness set will be determined by how m and f change with varying allocation of resources to male and female function (r and 1-r respectively, where r represents allocation to male function). Charnov (1982) plots male fitness *vs* r and female fitness *vs* 1-r; these are referred to as the male and female gain curves. Lloyd (1984 b) discusses these, and shows that a linear gain curve indicates that an increased allocation of resources is accompanied by an equivalent increase in individual fitness. However, a gain curve may also obey the laws of diminishing or increasing returns. When obeying the former, the increase in fitness obtained through either sex function is greater for the first resources put into that function than for further resources. When obeying the latter, the opposite is true. Charnov (1982) predicts that an hermaphrodite will only be favoured when the law of diminishing returns is obeyed by both gain curves. The relative allocations will depend upon the relative degree of non-linearity of the curves. Lloyd (1984 b), in his discussion, shows that more resource will be expended on the sex function with the slower rate of diminishing returns.

One factor which may result in different male and female gain curves is the occurrence of selfing in a population. Charlesworth and Charlesworth (1981) discuss the effect of selfing with inbreeding depression upon the relative allocation to male and female function in hermaphrodites. Using an ESS approach, they predict that, generally, as selfing increases, so allocation to male function decreases, although it may not necessarily

decrease to below 0.5. When considering inbreeding depression with selfing (δ indicating the level of inbreeding depression) they find that hermaphroditism is more stable with $\delta < 0.5$ and less stable with $\delta > 0.5$. Bulmer and Taylor (1980), Goldman and Willson (1986) and Lloyd (1984 b) also predict that increased selfing will result in decreased allocation to male function. Support for this prediction is provided by studies on *Ipomopsis rubra* (Benjamin and Hainsworth, 1986), *Bromus* species (McKone, 1986) and by Charnov (1982).

With increased selfing there will be increased competition between related pollen grains. This situation is very similar to that of local mate competition (LMC). LMC occurs in mating groups which may be small or have localised dispersal so that related individuals/gametes are competing with each other for mates. Hamilton (1967) developed the theory behind LMC. He predicted that a female should only produce enough sons to fertilize her daughters. Similarly, an hermaphrodite plant should only produce enough pollen to fertilize its seeds. Charnov (1982) shows that the ESS allocation to male and female function maximizes at the product relation:

$$f.m^{[(n-1)/(n+1)]}$$

where f is female function, m is male function, and n is the size of the mating group. The exponent $(n-1)/(n+1)$ shows how sons are devalued as a function of mating group size relative to daughters,

and thus shows how a female who could alter the number of sons produced in different group sizes could be favoured.

The theory that investment in male function will decrease as a result of LMC is based on the assumption that male function is limited by access to mates, and female function is limited by resources (Bateman's principle - Bateman, 1948, quoted from Charnov, 1982). Under this assumption, males will be competing with each other for mates. This theory has received widespread support (e.g. Bawa and Beach, 1981; Charnov, 1979; Lloyd,1979; Stephenson, 1984). However, Goldman and Willson (1986) provide some evidence that male function may be resource limited and female function limited by the availability of acceptable mates.

LMC may result from localised dispersal of offspring so that individuals in a small area are genetically related. Fisher (1930 - quoted from Charnov, 1982) in his prediction of a 1:1 sex ratio, assumed random mating. This assumption is invalid when localised dispersal of gametes or offspring occurs.

Bulmer and Taylor (1980) consider the sex ratio when offspring disperse to new patches, random mating occuring within patches. They conclude that selection will favour a sex ratio which is biased towards the sex which disperses more widely or more evenly. This is because sibs of the sex which disperses more widely will be less likely to be in the same patch as each other than will sibs of the sex that disperses less widely.

Consequently, the former will be less likely to be involved in LMC than the latter. The authors extend the model to look at resource allocation in hermaphrodites. They assume that pollen is dispersed between patches where it fertilizes available seed. The seed is then dispersed to another patch, in each of which only one seed succeeds in giving rise to a mature plant. Under these assumptions, they once again demonstrate that the sex ratio will be biased towards the sex function which disperses further or more evenly. Bulmer (1986) considers the sex ratio under Wright's island model of dispersal (Wright, 1943). He considers a gene locus with two alleles, R and S, and calculates gene frequencies for haploid, diploid, and haplo-diploid individuals under different rates of dispersal and with different types of gene action (S being dominant, recessive or additive to R). He does this for mating before and after dispersal. He concludes that with mating before dispersal, the dispersal rate has little or no effect on gene frequency; but with mating after dispersal, there is bias towards the sex with the higher dispersal rate.

Lloyd (1984 b) argues that if the pollen parent can fertilize only a limited number of other plants, an increase in pollen production will be associated with a decrease in the fitness gain curve. He also examines the effect of local seed dispersal (local resource competition - LRC). He concludes that, when the seed shadows of the various mates of a pollen plant do not overlap, LRC will result in a decrease in female function relative to male function. This is because an increase in female

function will result in increased competition between sibs for resources, causing the female gain curve to decrease more rapidly than the male gain curve.

LMC theory can be used to predict that male biased sex ratios should be most common among wind-pollinated plants. The male gain curves of anemophilous species should decrease more slowly than those of species exhibiting other methods of pollen dispersal (Charlesworth and Charlesworth, 1981; Goldman and Willson, 1986). This is because air-dispersed pollen from one individual will be intermixed with that from others, resulting in this type of pollen being dispersed more evenly than, say, insect-dispersed pollen. As a result, there will be less intra-individual competition between pollen grains of anemophilous species than between grains of species with other dispersal mechanisms.

Charnov (1982) strongly supports the LMC theory, basing his support on a wide range of data, including those presented by Cruden (1977) and Cruden and Miller-Ward (1981). Cruden (1977) determined pollen:ovule (P/O) ratios for a number of plant species (P/O being used as a measure of relative allocation to male and female function). He calculated the outcrossing indices for these species and then correlated P/O with the various breeding systems. He demonstrated that P/O decreased with an increased likelihood of pollination and consequently argued that P/O is an indicator of the breeding system. Cleistogamous flowers have the lowest P/O

($\bar{x}$ = 4.7), xenogamous species the highest ($\bar{x}$ = 5859.2). Cruden and Miller-Ward (1981) suggest that variation in the P/O reflects the efficiency of pollination, high efficiency resulting in low P/O and *vice versa*.

Charnov (1982) considers that variation in P/O may be due to LMC rather than to efficiency of pollination, as LMC predicts that allocation to male function will decrease as the incidence of selfing increases. Both theories seem to condense to this conclusion if examined carefully.

An individual's fitness gain curves may be altered depending upon the quality of the environment which it occupies. An environment may be 'patchy' with respect to quality (e.g. in availability of water, nutrients or light) and gain curves may vary between patches. A number of authors have proposed models which show that an individual which exhibits environmental sex determination (ESD - the ability of an individual to alter gender depending upon environmental conditions) may be favoured in a patchy environment (Bull, 1981; Charnov and Bull, 1977, 1985; Freeman *et al*, 1980). This labile sexuality is an example of the phenotypic plasticity in plants discussed by Bradshaw (1965, 1974) and Lloyd (1984 a).

Charnov and Bull (1985) conclude that selection will favour adjustment of a plant's gender in a particular patch if such an

adjustment increases its overall fitness (m.f). Bull (1981) used a dioecious system to reach a similar conclusion. He made the proviso that where there was existing heavy bias towards one sex, the allocation to that particular sex may not be selected for even if it was the sex most favoured in that environment. He also predicted that if the frequency of patch type varies between generations, there would be a greater tendency for a sex ratio of 1:1 to be favoured in each patch. Charnov and Bull (1977) conclude that ESD will be particularly favoured where an individual has little control over, or predictive ability of, the environment it occupies.

Charnov (1986) extends the theory of sex allocation to look at temporal variation in male *versus* female fitness, and again concludes that an individual that can increase its fitness in a temporally varying environment by altering its sex ratio will be selected for. Several authors have suggested that gender may vary with age or size of the plant (Charnov, 1982; Charnov and Bull, 1977; Freeman *et al*, 1980; Solomon, 1985). Since reproduction may be more costly through one sex than through the other (Freeman *et al*, 1980; Lovett-Doust and Cavers, 1982) growth and mortality rates may be sex specific. The organism will spend its early years in the sex with the lower mortality and/or the higher growth rate, and will delay reproduction by the sex which pays higher mortality/growth costs to a later age or larger size. Bierzyduchek (1981 - quoted from Charnov, 1982) showed that mortality rate was size dependent but not sex dependent in

Arisaema triphyllum. Policansky (1981), however, shows that female reproductive success (RS) increased with size, whereas male RS did not in this species. Lovett-Doust and Cavers (1982) showed that, on average, female *A. triphyllum* plants were larger than males (55 cm height *vs* 41 cm for one site, and 43 cm *vs* 33 cm for another). This theory may help to explain sequential hermaphroditism - the phenomenon whereby an individual reproduces as one sex early in life, then changes to another for the rest of its life.

Bawa (1980) and Bawa and Beach (1981) also explain the evolution of different sexual systems in plants in terms of the optimization of maternal and paternal investments and suggest that the observed patterns may arise mainly as a result of reproductive competition resulting from sexual selection (see also Willson, 1979). However, the crucial new element to their hypothesis is that the evolution of a particular system is controlled largely by the ecology of the pollination system. For example, hermaphrodite systems have an advantage over dioecious or monoecious systems when a pollinator is foraging for pollen only. This is because both male and female function are found in all flowers so the pollinator will visit all flowers, pollinating ovules as it collects pollen. If the sexes were separate, the pollinator would visit only male flowers. However, they have been unable to develop a general and predictive hypothesis.

There has been a lot of debate about how allocation to male and female function should be measured.

Bell (1985) proposed a method for calculating the 'gender' of secondary floral allocation (e.g. to petals, nectar, etc.). He suggests that such structures should not be considered as being equally male and female in terms of investment. Through observation and manipulation of flower size in five species, he concludes that 'the flower is primarily a male organ'. He shows that a single insect visit (or very few visits) is sufficient to pollinate almost all the ovules, whereas numerous visits are required to disperse available pollen. Charnov (1982), Goldman and Willson (1986) and Lloyd (1984 b) also examine this problem, but come to no theoretical basis for the partitioning of flowers. Consequently they consider the costs as being bilateral. Charnov and Bull (1986) investigate the effect of pollinator attractiveness of a plant on reproductive allocation and allocation to attraction structures for the dispersal of pollen. They conclude that, for an hermaphrodite to be stable, both male function and investment in attraction structures must obey the law of diminishing returns.

Goldman and Willson (1986) examine the various 'currencies' which can be used in determining allocation to the two sex functions. They criticise the use of biomass in these measures when no note is taken of compositional differences, since the energy content of materials varies with chemical composition, and

since the physiological costs of producing different compounds may vary. They also argue that it is difficult to know what may be limiting reproduction, e.g. carbon (or energy) or some other material (N, P, K, Ca, Mg, etc.), and therefore what currency should be studied. They suggest that measures of respiration provide the most complete estimate of reproductive effort when energy or carbon is the limiting resource. However, Lloyd (1984 b) suggests that final dry weights should be the 'currency' used.

The concept of 'functional gender' has been developed by Lloyd (1979, 1980). He suggests that it is difficult to measure directly the parental success of a plant by following the fate of all pollen or all seeds. However, it is possible to calculate the proportion of genes most likely to be transmitted through either male or female function, and thus measure the likely success of an individual as a mother or father. He derives equations for the calculations of numerical indices for describing the functional gender. First, he calculates an equivalence factor (E) by which male and female units can be related

$$E = \frac{\sum_i g_i}{\sum_i a_i}$$

where g_i = gynoecial production of plant (measured by ovule no., seed no., fruit no., or ovary no.)

a_i = androecial production of plant (measured by pollen grains, anthers or polliniferous flowers)

"E weights the androecial units so that they are commensurable with gynoecial units as a measure of fitness." This assumes that an androecial unit has the same probability of contributing genes to the next generation as a gynoecial unit. The functional femaleness (G_i) and maleness (A_i) can then be calculated as below:

$$G_i = \frac{g_i}{g_i + a_i E}$$

$$A_i = \frac{a_i E}{g_i + a_i E}$$

The limitation of these measures is that they do not give the actual investments of plants as sexual parents. However, they do give a good measure of the effective gender of the plant (Lloyd, 1979). They do not specify the relative amounts of resources allocated to male or female function. However, Bawa and Beach (1981) suggest that estimates of functional and effective gender are more valuable than those based on intrinsic estimates and morphological appearance.

The use of pollen:ovule ratios (P/O's) as a measure of gender is similar to Lloyd's method. However, it does not incorporate an equivalence factor to equate gynoecial and androecial units. Cruden (1977), Cruden and Miller-Ward (1981), and Queller (1984), all use P/O's in the study of sex allocation. However, Queller (1984) suggests that investment of resources in pollen and ovules, in addition to the number of pollen and ovules,

should also be taken into account. The former cannot always be measured, as has been discussed.

The timing of decisions on parental investment may have a large effect on the investment in either reproductive function. Lloyd and Bawa (1984) suggest four means of modifying gender: sterilization or not of stamens or pistils; a dichotomous decision between sex functions; pollen:ovule emphasis in a flower; abortion or non-abortion of seeds. The timing of decisions on paternal investment is concentrated in a very short period, since paternal expenditure is brief (Lloyd, 1979). However, maternal investment continues from flower initiation until maturation of fruit or seeds. Maternal investment may be modified at three stages: flower determination; ovary development; fruit maturation (Lloyd, 1979, 1980 c; Lloyd *et al*, 1980). The choice between the first two stages and the third depends upon the extent to which the success of seed maturation can be predicted (Lloyd, 1979). Lloyd (1980 c) hypothesizes that initiation and continuation of investment requires a threshold amount of available resources at each stage and that the pattern of control that maximizes maternal fitness will be selected for. Lloyd *et al* (1980) provide circumstantial evidence from seventeen angiosperm species to support the hypothesis of serial adjustment of maternal investment.

Stephenson (1984) shows that flower number and seed yield in *Lotus corniculatus* are regulated during reproduction in response

to resource availability. He suggests that flowers which fail to produce mature fruit could either be functioning as males or providing plants with a choice of fruits to mature. This first suggestion is supported by Sutherland and Delph (1984), who argue that the optimal number of male flowers in hermaphrodites is greater than the optimal number of female flowers and, therefore, that male function is the ultimate factor in determining total flower production. This results in the apparent over-production of ovaries and in low seed set in hermaphrodite plants compared with plants in which the numbers of male and female flowers can be adjusted independently. Sutherland (1986) presents evidence from 447 species of plants to support this hypothesis.

The method by which plants control the relative allocation to male and female function has not been studied in great detail. The rôle of plant hormones has, however, been discussed by a number of authors. Freeman *et al* (1980) review some of the evidence. They conclude that at least some plants possess "environmental monitoring systems" which allow them to adjust sex expression. They give a list of known hormones (and environmental conditions) which have been shown to alter gender in particular species. They also present evidence that certain environmental conditions cause changes in hormone levels which may, in turn, bring about changes in sex expression. Their evidence is mostly circumstantial, and further investigations need to be completed before their conclusions can be accepted.

Freeman and McArthur (1984) suggest that phytohormones may have a rôle in ESD, and since sex genes ultimately control the production of phytohormones (Louis and Durand, 1978, quoted from Freeman and McArthur, 1984), it does not seem surprising that the two may be linked.

A major aim of this project has been to discover at least the basis for sex allocation in *R. bulbosus*. Does it have a purely genetic 'fixed' basis, or can it exhibit ESD? This has been investigated by examining pollen:ovule ratios in a natural population, and by carrying out experiments to show the response of *R. bulbosus* plants (in terms of sex allocation) to stress.

3.2 EXPERIMENTAL DESIGN AND RESULTS

In this section, the general methods used will first be described. An account of each experiment will follow and the results will then be presented and discussed. The Chapter will be concluded with a general discussion.

3.2.1 GENERAL METHODS

a) Pollen Counts

Pollen counts were always carried out on semi-open buds before anther dehiscence (Fig. 3.3).

In order to obtain accurate pollen counts, a reliable extraction method was required. This was provided by Dr. John Piper (pers. comm.). It involved dissolving the anther wall by heating the anthers in acid. This left intact (although inviable) pollen grains in suspension. The extraction method is described below:

1. A solution of acetic anhydride and concentrated sulphuric acid was prepared in a ratio of 9:1 (v/v) respectively. The solution does not keep, and was prepared fresh for each set of preparations. When mixing the solutions, the beaker was kept cool

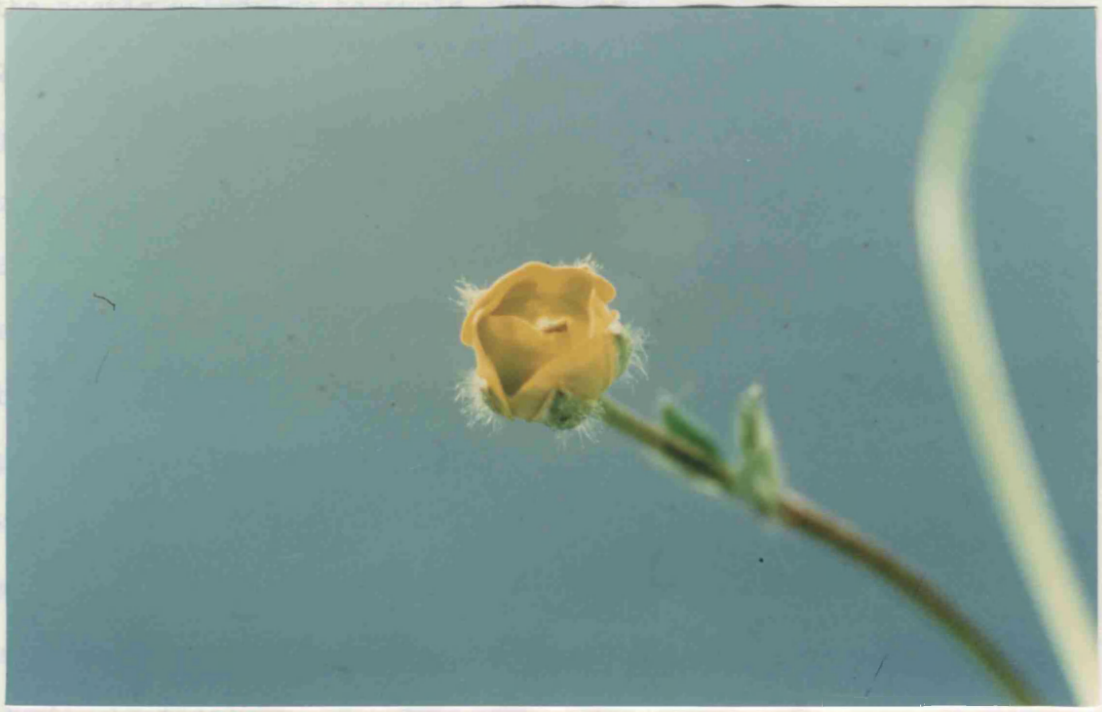


FIG. 3.3 A semi-open bud before anther dehiscence

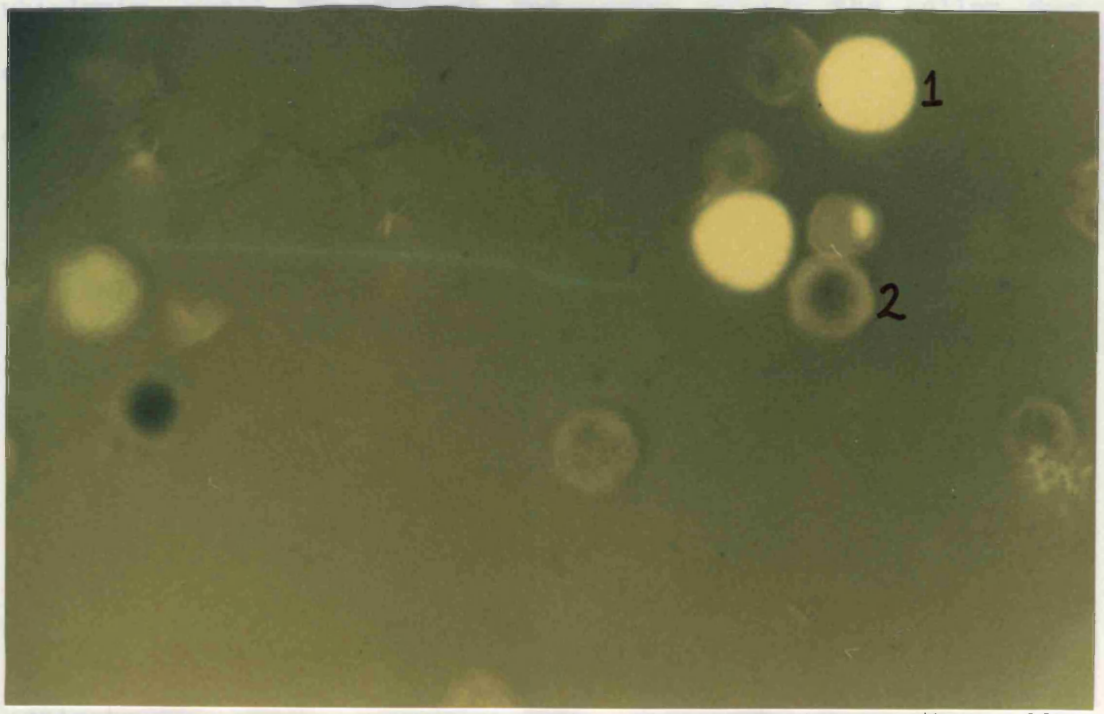


FIG. 3.5 Pollen stained using FDA. Brightly fluorescing pollen grains (1) are viable. Dull pollen grains (2) are not viable.

(by placing it in an ice bath) and the acid was always added to the acetic anhydride to avoid explosions!

2. 0.2 ml aliquots of the acid solution were pipetted into graduated ependorfs and the level of liquid marked on the outside of the ependorf.

3. Six anthers were placed in each filled ependorf. The ependorfs were then floated on the surface of water in a water bath set at 80 °C. The lids of the ependorfs were left open to prevent a build-up of pressure.

4. After 45 minutes (by which time the anther wall was softened) the ependorfs were taken out of the water bath and the solution topped up to the mark (the liquid tends to evaporate during heating).

5. The contents of the ependorfs were shaken, using a Fisons whirlymix, until the anthers had broken up and the pollen was dispersed through the solution.

6. 0.3 ml aliquots of a 50% glycerol solution were added to the contents of each ependorf and the contents mixed using a Fisons whirlymix.

The number of anthers that needed to be used to provide a reliable estimate of the number of pollen grains per flower was determined by calculating the running mean of pollen number from single anthers. An investigation of flowers from ten different plants showed that six anthers were sufficient to provide this estimate.

Several methods for counting the pollen in solution were investigated. Since there was to be a large number of samples counted, the quickest method of obtaining an accurate count was required.

i) Counting by eye

It was not possible to count pollen grains in the concentrations in which they were found. Samples were, therefore, diluted by a factor of 1 in 10 using 50% glycerol solution. This reduced the concentration of pollen grains to an assessable level. Tubes were shaken on a whirlymix to distribute the pollen grains as evenly as possible through the solution. Five 10 µl aliquots were taken from each tube and pipetted onto five glass microscope slides. The microscope slides had previously been marked, using a diamond pencil, with a grid pattern to help in counting. The liquid was pipetted in such a way as to make a straight line along the grid (Fig. 3.4).

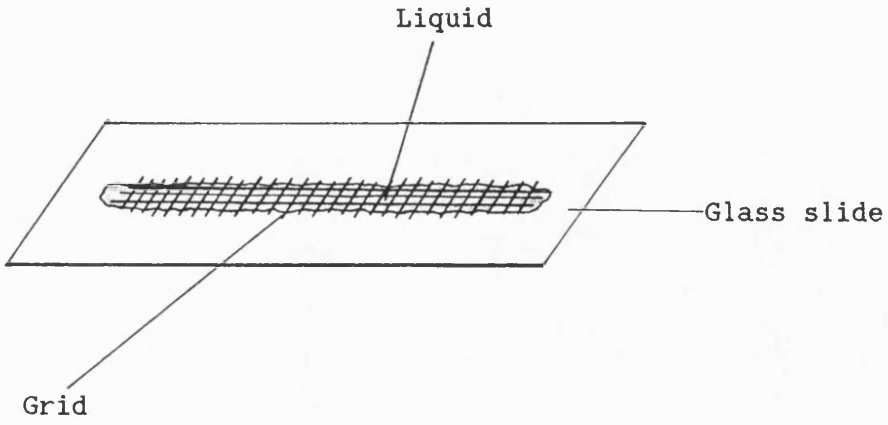


FIG. 3.4 Diagram to show the positioning of liquid on a marked glass slide.

The number of pollen grains per 10 µl aliquot was then counted using the X400 lens on a light microscope. The average number of pollen grains per 10 µl aliquot was calculated and multiplied by a dilution factor to find the amount of pollen per anther. Five 10 µl aliquots were found to be adequate to give an estimate of the true mean using the running mean technique described above. Once the amount of pollen per anther had been found, this was multiplied by the total number of anthers in the flower to give an estimate of total pollen per flower.

ii) Estimating pollen concentration using optical density

Optical density, measured using a spectrophotometer, is often used to estimate the concentration of algae in solution (Clarke, C.A., pers. comm.). Such a method could prove useful for estimating pollen concentration.

Absorption spectra for wavelengths between 100 nm and 650 nm were obtained for all the component parts of the pollen solution and for the pollen solution itself. Peaks were found at 496 nm for pollen in water and at 261 nm and 343.6 nm for the pollen solution. No peaks were found for the other solutions.

Pollen from 20 samples was counted by eye. These samples were prepared using only one anther, so that no dilutions had to be performed. Absorption of the same 20 samples was measured at four wavelengths (300 nm, 496 nm, 500 nm and 600 nm). No relationship was found between counts carried out by eye and

absorption at any of the wavelengths used. This was also found to be the case when the acid/glycerol solution was removed and the pollen resuspended in 0.5 M sucrose solution.

Transmission spectra were also investigated, but there was no relationship between the estimate obtained by this technique and that obtained from counting by eye.

iii) Counting pollen grains using a cell counter

Samples of pollen suspended in acid/glycerol solution were prepared as previously. Pollen grains were then spun down using a bench centrifuge and the supernatant removed. The pollen grains were then resuspended in 0.9% NaCl, 0.1% NaN_3 solution (this had previously been filtered, so as to be non-particulate). The samples were sonicated for ten seconds on low frequency to give an even suspension. Samples were then counted on the coulter counter. The relationship between these counts and those counted by eye was not sufficiently strong to warrant the use of the coulter counter in counting pollen grains.

I suggest that the presence of particulate matter from the digested anther walls was responsible for the differences found between samples counted by eye and those counted by other methods. This particulate matter cannot be distinguished from pollen by any of the above methods other than that involving counting by eye. It was, therefore, necessary to count pollen grains by eye. This decision was reached with some regret, as counting by eye is by

far the most time-consuming method. This meant that fewer pollen counts could be carried out in the time available.

b) Ovule Counts

In *R. bulbosus* each ovule is contained in a separate carpel, each of which is between 1 - 2 mm diameter before fertilization. Therefore, in order to accurately estimate ovule number, it is necessary only to count the number of carpels, which are easily visible under a dissecting microscope.

c) Pollen Viability

Not all pollen contained in anthers is viable. To obtain estimates of viable pollen number it was necessary to determine a technique to measure viability. Several were investigated:

i) Fluorescein diacetate stain (Heslop-Harrison and Heslop-Harrison, 1970)

1. A stock solution of 5 mg of fluorescein diacetate (FDA) powder in 1 ml acetone was prepared. This was kept in the dark in a freezer, under which conditions it should stay active for 2-3 weeks.

2. When required, a working solution was prepared by adding stock solution drop by drop to approximately 2 ml of 0.5 M sucrose solution (3.423 g/10 ml water) until the mixture turned opaque.

This solution was kept on ice in the dark, under which conditions it should stay active for up to 2 hours.

3. As much pollen as possible was extracted from an anther onto a clean glass slide. Two drops of FDA were added to the pollen.

4. The slide was placed in a darkened petri dish (covered with black paint) which had been lined with moist filter paper, and left for 30 minutes at room temperature.

5. The stained pollen was observed using an Olympus ultra-violet microscope, using a 570 barrier filter (which takes out light of wavelength <570 nm) and a blue excitor filter (which transmits light between 390-475 nm). Viable pollen grains showed bright yellow fluorescence. Non-viable grains were dull. See Fig. 3.5.

ii) Germination of pollen tubes in Kwak's liquid medium

1. Kwak's liquid medium was prepared by dissolving the following ingredients in 100 ml of distilled water:

0.01 g boric acid (100 ppm)

0.03 g calcium nitrate (300 ppm)

0.02 g magnesium sulphate (200 ppm)

0.01 g potassium nitrate (100 ppm)

0.5 ml Tween 80 (wetting agent)

Sucrose was added to the medium at concentrations of 10, 20, or 30 g/100 ml.

2. Pollen was extracted from an anther in a drop of Kwak's liquid medium on a clean glass slide.

3. The pollen and liquid were washed off the slide into a small plastic petri dish and mixed to disperse the pollen.

4. Three drops of the pollen suspension were placed in each of four wells of a 35 mm tissue culture petri dish (Sterilin products, Cat. No. 316).

5. The dish was sealed with parafilm and placed in a box lined with moist tissue paper, to prevent desiccation.

6. The boxes containing the dishes were placed in incubators set at either 25 °C or 30 °C and left for 18 hours.

7. The percentage of viable pollen grains was estimated by counting total number of pollen grains and the number of pollen grains possessing a pollen tube. Counts were made at five random points in each tissue culture well. The mean was then calculated for each well and for the whole dish.

iii) Germination of pollen tubes on agar drops surrounded by varying concentrations of sucrose solution (Böcher, 1938)

1. Four drops of 0.6% agar were placed in a small plastic petri dish.

2. Once the agar had dried, pollen was brushed directly from an anther onto the surface of the agar.

3. Sucrose solution was poured carefully into the petri dish until the agar drops were covered. Different concentrations (10, 20 or 30%) of sucrose were used.

4. The petri dishes were sealed with parafilm and incubated at either 25 °C or 30 °C for approximately 18 hours.

5. The surfaces of the agar drops were scanned using a light microscope (x100 magnification), and the proportion of pollen that had germinated was estimated.

iv) Germination of pollen tubes on Kwak's solid medium using different sucrose concentrations and incubation temperatures

1. Kwak's solid medium was prepared as per method ii), with the addition of 0.7 g of lab 'm' agar mc2 and the omission of the Tween 80.

2. The surface of a clean microscope slide was thinly coated with the medium and left to dry.

3. Once the agar had dried, a dehiscing anther was brushed over its surface so that a layer of pollen was deposited.

4. Prepared slides were placed in a box lined with moist tissue paper (to prevent desiccation) and incubated at 25 °C or 30 °C for approximately 18 hours.

5. Five random points on the surface of the agar were selected. The total number of pollen grains and the number of grains with pollen tubes within a field of vision at each point were counted.

v) Germination of pollen tubes on the surface of Kwak's liquid medium

1. As for method ii).

2. Three drops of the medium were placed in each of four wells of a 35 mm tissue culture petri dish.

3. The pollen was extracted from an anther onto a clean glass slide and, from there, sprinkled onto the surfaces of the drops of medium.

4. Petri dishes were sealed with parafilm, placed in a box lined with moist tissue paper, and incubated at either 25 °C or 30 °C for 18 hours.

5. The surfaces of the drops of medium were scanned using a light microscope (x100 magnification).

The pollen tube germination methods tested gave very poor values of estimated pollen grain viability (between 0% and 9%). The estimates using the FDA stain were higher (60%-100%) and showed some variation within and between plants. This method was consequently used to estimate pollen viability.

d) Seed Set

The ovules of *R. bulbosus* are each contained in a separate carpel. Following fertilization, the carpel swells and its coating hardens and darkens. To estimate percentage seed set, therefore, it is necessary only to count the number of swollen, darkened carpels, and the total number of carpels.

e) Rosette Size

Before flowering, the above-ground parts of *R. bulbosus* consist of a rosette of leaves (see Fig. 3.6). The size of the rosette was estimated by taking the mean of its two longest and two shortest diameters. These were selected by eye. The mean was then multiplied by the total number of leaves contained in the rosette. This gave a quantitative and comparable estimate of rosette size.

f) Corm Size

After flowering, all above-ground parts of the plant die, leaving the corm in the ground. Corm size was estimated as dry weight, the corm being collected once all above-ground parts of the plant had died. A constant dry weight was obtained after the corm had been subjected to a drying temperature of 70 °C for 168 hours.

g) Competing Plants

Two plants, when grown in close proximity, may compete with each other for finite resources. Competition is shown to occur if one or both of the plants is more successful when grown on its own. To try to achieve competition between two plants, an 'experimental' plant was allowed to establish itself. A second plant of the same genotype was then planted within a 4 cm radius

Fig 3.6 R.bulbosus consists of a small rosette of leaves before flowering - this plant has just produced its' first flower bud (1)

of its centre. The two plants were left *in situ* until the end of the flowering season.

h) Depriving a Plant of Nutrients

A recently cloned plant was removed from its pot and all excess soil washed away. The plant was then potted in a 3.5" pot filled with coarse sand and watered well. No other nutrients were supplied to the plant.

i) Waterlogging a Plant

A recently cloned plant was potted in a 3.5" pot filled with Fison's M2 compost and watered well. The pot was then stood in a tray filled with water which was kept topped up so as to keep the plant waterlogged.

3.2.2 EXPERIMENTS PERFORMED

a) A field study of anther and ovule numbers in a population of *R. bulbosus* at Rainbow Wood Meadow

i) Methods

A random sample of 120 flower buds was collected from Rainbow Wood. The numbers of anthers and ovules each bud contained were counted. A regression analysis was performed on anther *versus* ovule number using the Minitab statistical package.

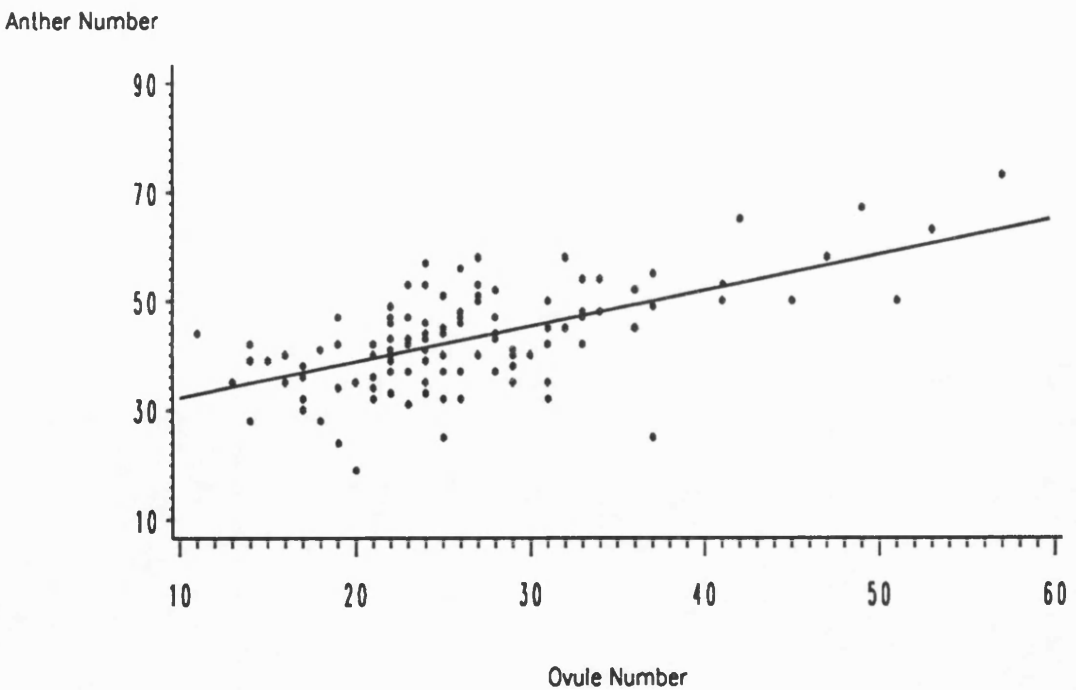
ii) Results

The results are presented graphically in Fig. 3.7. The regression analysis showed a highly significant positive linear regression ($F = 68.74$, $p < 0.001$) of anther number per flower on ovule number per flower. That is, the number of anthers per flower increases in a linear fashion with an increase in the number of ovules per flower.

iii) Discussion

The results suggest that there is variation in allocation ratio within the population at Rainbow Wood Meadow. However, the source of this variation is not revealed in this experiment. Each count of anther and ovule number per flower was from a different plant. The plants from which flowers were collected were widely spread throughout the field which exhibits a variety of different habitats. So, the difference exhibited here may have been

Fig. 3·7 – Anther And Ovule Number For 120 Flowers From A Population Of R.Bulbosus At Rainbow Wood, Bath



intrinsic differences between plants or (if *R. bulbosus* exhibits ESD) differences in allocation strategies between plants occupying different habitats.

An examination of Fig. 3.7 suggests that the significant positive relationship may tend towards being curvilinear. Overall, it appears that allocation to male function increases more rapidly than allocation to female function at lower levels of total allocation and *vice versa* at higher levels of total allocation. This could be explained in terms of the theory discussed in the introduction.

A plant may be allocating less to reproduction because it is small or growing in poor conditions and so has less resources available overall. It has been predicted that such a plant will allocate more of its resources to male reproductive function. Similarly, a plant may be allocating more to reproduction because it is big or growing in favourable conditions, with more resources available overall. It has been predicted that such a plant will allocate more of its resources to female reproductive function. This would account for the apparent change in allocation strategy with change in total allocation to reproduction. However, this is only speculation since, in this preliminary survey, nothing is known about the overall status of the plant, the number of the selected flower in the flowering sequence, or the conditions in which the plant is growing.

The use of anther number provides only a rough estimate of allocation to the male reproductive function. Further experiments showed that the number of pollen grains contained in an anther can vary considerably between flowers. In subsequent experiments, therefore, it was decided to use pollen number, rather than anther number, as a measure of male reproductive function.

b) A study of allocation to male and female gametes in five greenhouse plants

i) Methods

Five plants, collected randomly from Rainbow Wood, were kept in an unheated greenhouse throughout the Spring/Summer of 1986. They were visited every day during the flowering season and any partly-opened buds (Fig. 3.3) were removed. These were used to obtain estimates of ovule number, total pollen number, and the number of viable pollen grains. Kruskal-Wallis non-parametric tests were performed to test for differences between plants in mean ovule number, total pollen count (tpollen), viable pollen count (vpollen), total pollen:ovule ratio (tratio) and viable pollen:ovule ratio (vratio). This test was used because the data were non-normal and could not be transformed to normality. Regression analyses against time were performed. Time was calculated as being 'own' time. 'Own' time was calculated separately for each plant, starting from the first day of its own flowering season. Regression analyses were performed separately for each plant on ovule number, tpollen, vpollen, tratio, and vratio, against 'own' time. All statistics were performed using the Minitab statistical package.

ii) Results

The Kruskal-Wallis tests showed significant differences between plants for ovule number, tpollen and tratio. The results are presented in tables 3.1 to 3.5.

TABLE 3.1

TABLE OF MEAN OVULE NUMBER BY PLANT WITH RESULTS OF KRUSKAL-WALLIS TEST BETWEN MEANS

	PLANT 1	PLANT 2	PLANT 3	PLANT 4	PLANT 5
MEAN	31.29	37.00	40.01	38.41	52.31
STANDARD DEVIATION	4.27	8.33	7.53	4.35	5.73
SAMPLE SIZE	7	19	13	41	29

Kruskal-Wallis test: H = 60.15 ($p < 0.05$).

TABLE 3.2

TABLE OF MEAN TPOLLEN BY PLANT WITH RESULTS OF KRUSKAL-WALLIS TEST BETWEEN MEANS

	PLANT 1	PLANT 2	PLANT 3	PLANT 4	PLANT 5
MEAN	846920	2060240	1603639	1234452	1470358
STANDARD DEVIATION	327676	626792	419867	331658	252840
SAMPLE SIZE	5	16	11	40	29

Kruskal-Wallis test: H = 33.45 ($p < 0.05$).

TABLE 3.3

TABLE OF MEAN VPOLLEN BY PLANT WITH RESULTS OF KRUSKAL-WALLIS TEST BETWEEN MEANS

	PLANT 1	PLANT 2	PLANT 3	PLANT 4	PLANT 5
MEAN	386308	914839	1018060	894381	1064480
STANDARD DEVIATION	76872	435948	470764	431551	446023
SAMPLE SIZE	4	13	8	33	26

Kruskal-Wallis test: H = 9.029 ($p > 0.05$).

TABLE 3.4

TABLE OF MEAN TRATIO BY PLANT WITH RESULTS OF KRUSKAL-WALLIS TEST BETWEN MEANS

	PLANT 1	PLANT 2	PLANT 3	PLANT 4	PLANT 5
MEAN	28848	57054	41507	31721	28265
STANDARD DEVIATION	11289	14632	9174	6480	4807
SAMPLE SIZE	5	16	11	40	29

Kruskal-Wallis test: H = 49.45 ($p < 0.05$).

TABLE 3.5

TABLE OF MEAN VRATIO BY PLANT WITH RESULTS OF KRUSKAL-WALLIS TEST BETWEN MEANS

	PLANT 1	PLANT 2	PLANT 3	PLANT 4	PLANT 5
MEAN	13678	25441	26978	22604	20188
STANDARD DEVIATION	2638	12179	12781	10485	8204
SAMPLE SIZE	4	13	8	33	26

Kruskal-Wallis test: H = 7.68 ($p > 0.05$).

The regression analyses by plant for all variables against 'own' time showed a significant negative linear relationship in ovule number with time for plants 1-4. There was no significant difference between the slopes of the regression for these 4 plants ($F = 1.98$, $p > 0.05$). This relationship was not significant for plant 5. Total pollen was also found to exhibit a significant negative linear relationship with time for plants 2 and 4. There was no significant difference between the slopes of the regressions for these 2 plants ($F = 1.11$, $p > 0.05$). In fact, a significant negative linear relationship was found for all variables with time for plant 4. A summary of the results is contained in table 3.6. Where a significant result was obtained from the regression analyses, the results have been plotted. These plots are displayed in Figs. 3.8 to 3.16.

iii) Discussion

The sample sizes varied greatly between plants and were very small for some plants (plant number 1 in particular). This means that only limited conclusions can be drawn. Unfortunately, it was not possible to measure seed set, since all of the flowers produced were collected for analysis. However, the experiment was performed simply to determine whether *R. bulbosus* was a suitable vehicle to use in the study of sexual allocation.

All plants were propagated and kept under similar controlled conditions in a greenhouse. This should have minimized any environmental effects which may have caused variation between

TABLE 3.6 REGRESSION ANALYSES FOR ALL MEASURED VARIABLES AGAINST 'OWN' TIME CARRIED OUT SEPARATELY FOR EACH PLANT

PLANT NO.	FACTOR	REGRESSION COEFFICIENT	F	t	SIGNIFICANCE
PLANT 1	OVULE	-0.96	41.17	-6.42	p <0.01
	tPOLLEN	22772	0.18	0.43	p >0.05
	vPOLLEN	107438	0.05	0.22	p >0.05
	tRATIO	1320	0.61	0.78	p >0.05
	vRATIO	31	1×10^{-4}	0.01	p >0.05
PLANT 2	OVULE	-0.92	38.9	-6.24	p <0.01
	tPOLLEN	-54703	9.16	-3.03	p <0.01
	vPOLLEN	-6646	0.09	-0.3	p >0.05
	tRATIO	-487	0.85	-0.92	p >0.05
	vRATIO	-232	0.14	0.38	p >0.05
PLANT 3	OVULE	-1.30	23.62	-4.86	p <0.01
	tPOLLEN	-39751	1.77	-1.33	p >0.05
	vPOLLEN	-50506	1.14	-1.07	p >0.05
	tRATIO	-92	0.02	0.13	p >0.05
	vRATIO	-241	0.03	-0.17	p >0.05
PLANT 4	OVULE	-0.91	30.93	-5.56	p <0.01
	tPOLLEN	-54516	13.09	-3.62	p <0.01
	vPOLLEN	-72442	8.67	-2.94	p <0.01
	tRATIO	-713	4.92	-2.22	p <0.05
	vRATIO	-1612	6.95	-2.64	p <0.05
PLANT 5	OVULE	-0.37	1.23	-1.11	p >0.05
	tPOLLEN	-18516	1.61	-1.27	p >0.05
	vPOLLEN	4083	0.02	0.14	p >0.05
	tRATIO	-131	0.21	-0.46	p >0.05
	vRATIO	258	0.24	0.49	p >0.05

Fig. 3·8 – A Plot Of Variation In Ovule Number With Time For Greenhouse Plant 1

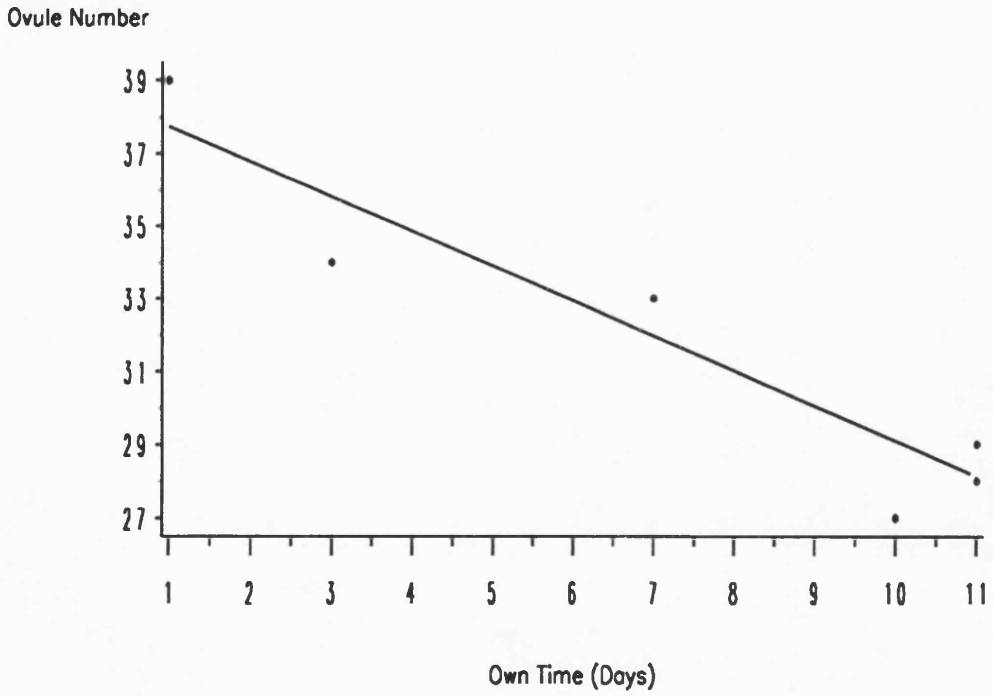


Fig. 3.9 – A Plot Of Variation In Ovule Number With Time For Greenhouse Plant 2

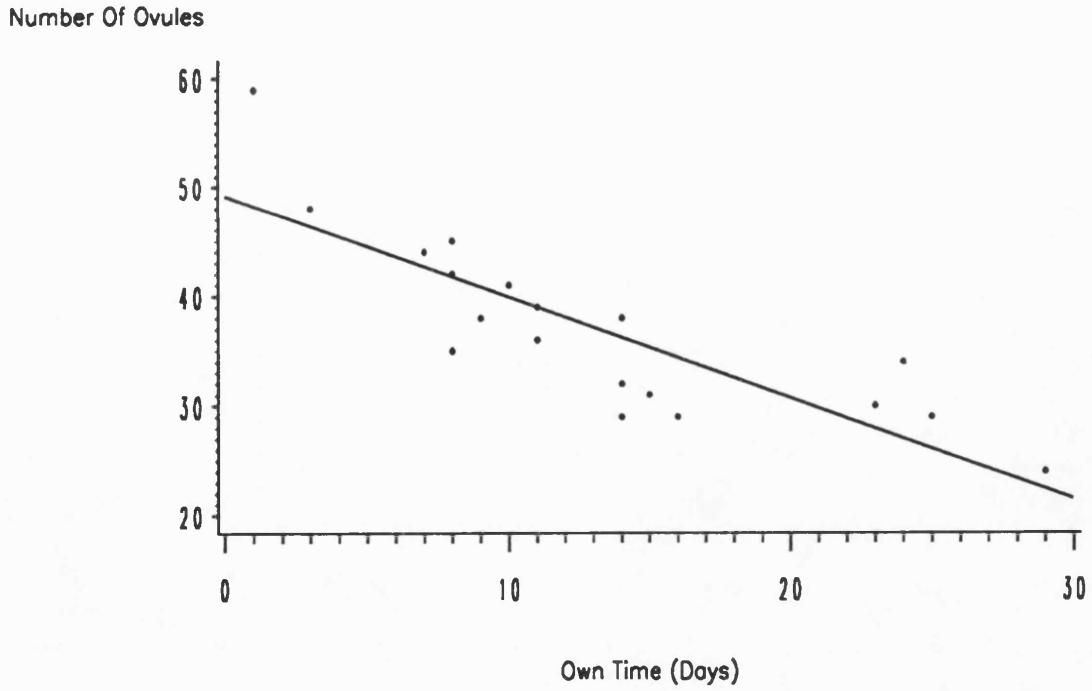


Fig. 3·10 – A Plot Of Variation In Ovule Number With Time For Greenhouse Plant 3

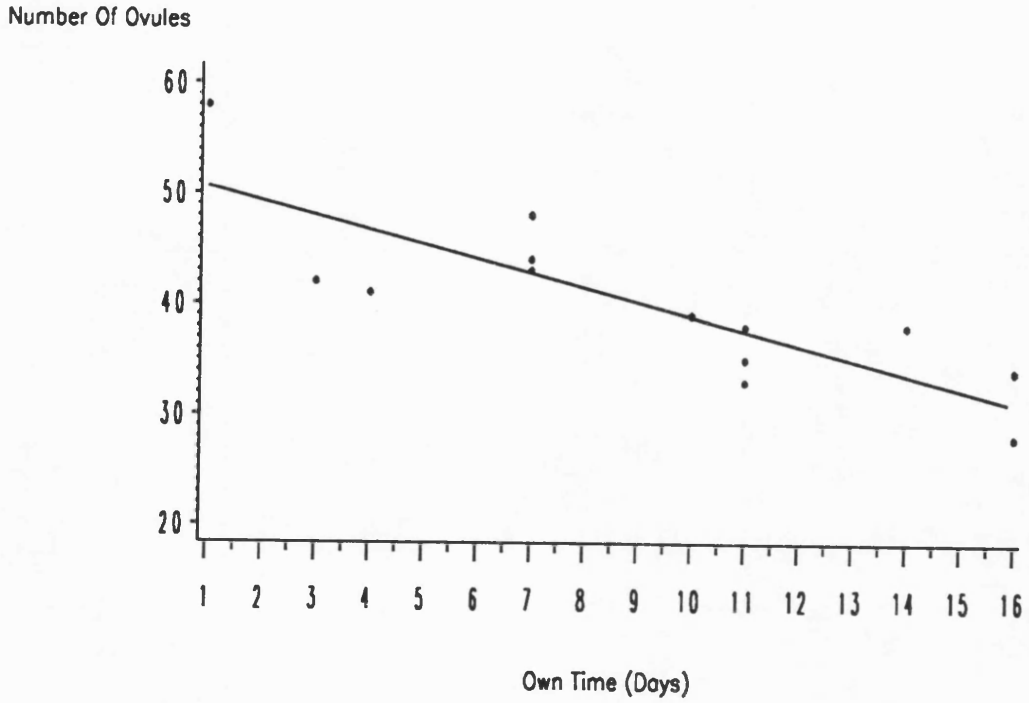


Fig.3·11 – A Plot Of Variation In Ovule Number With Time For Greenhouse Plant 4

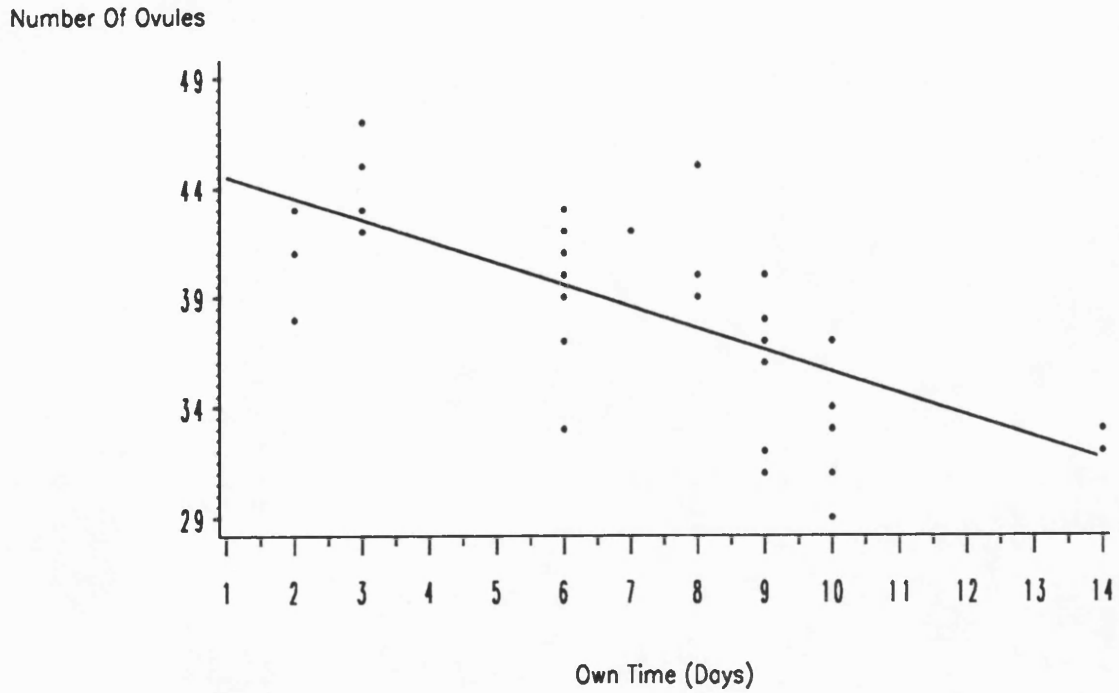


Fig. 3.12 – A Plot Of Variation In Total Pollen Count With Time For Greenhouse Plant 2

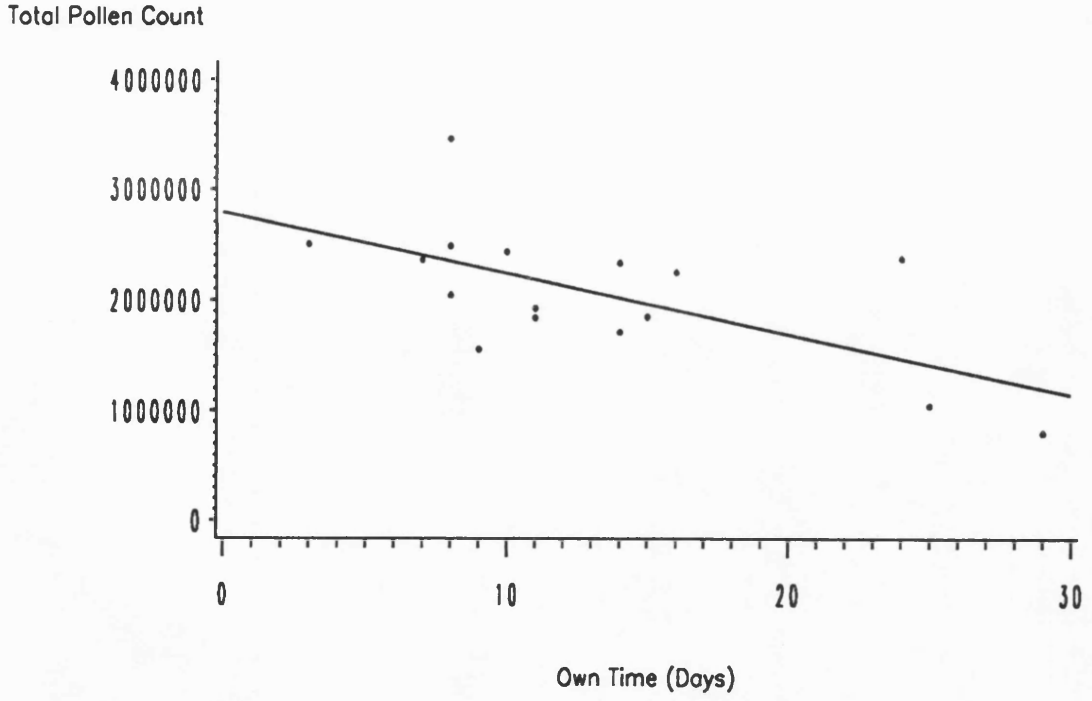


Fig.3·13 – A Plot Of Variation In Total Pollen Count With Time For Greenhouse Plant 4

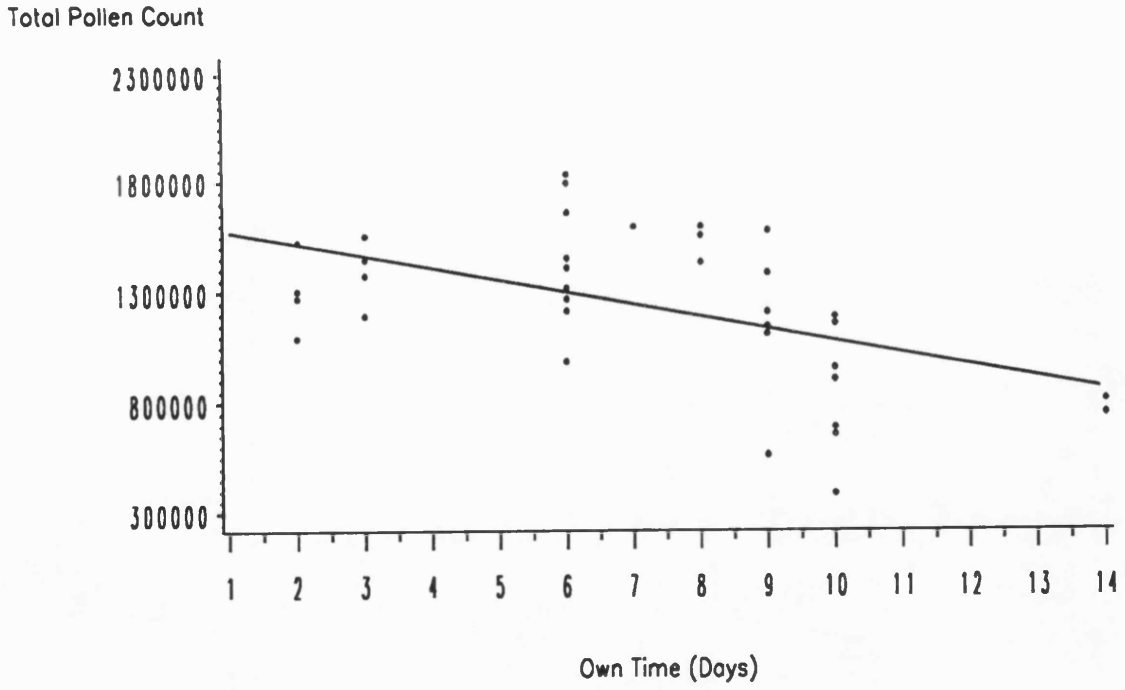


Fig.3.14 – A Plot Of Variation In Viable Pollen Count With Time For Greenhouse Plant 4

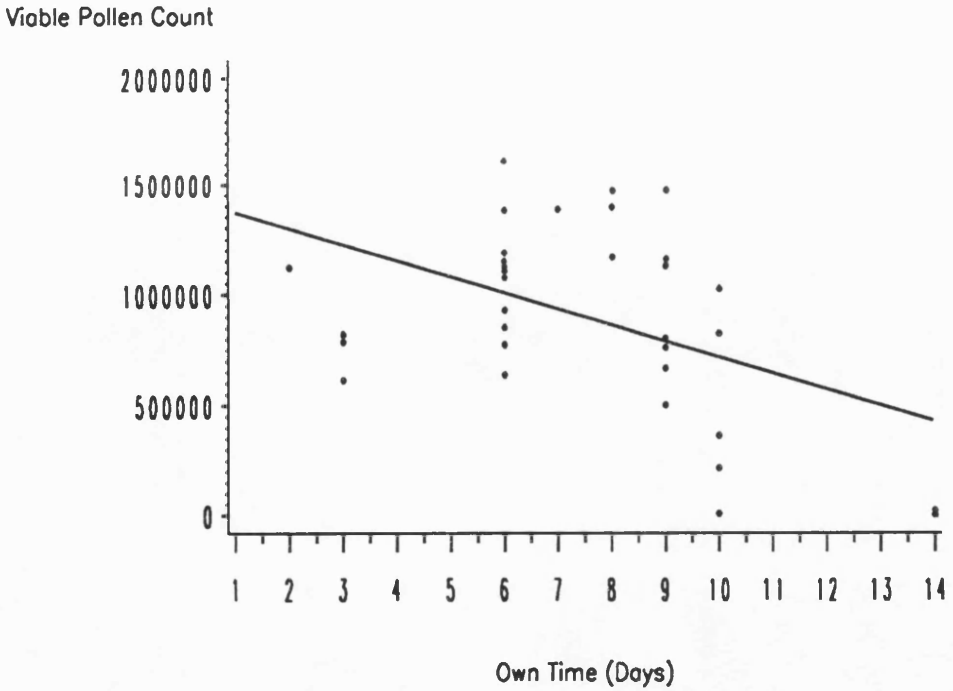


Fig. 3.15 – A Plot Of Variation In Total Pollen : Ovule Ratio With Time For Greenhouse Plant 4

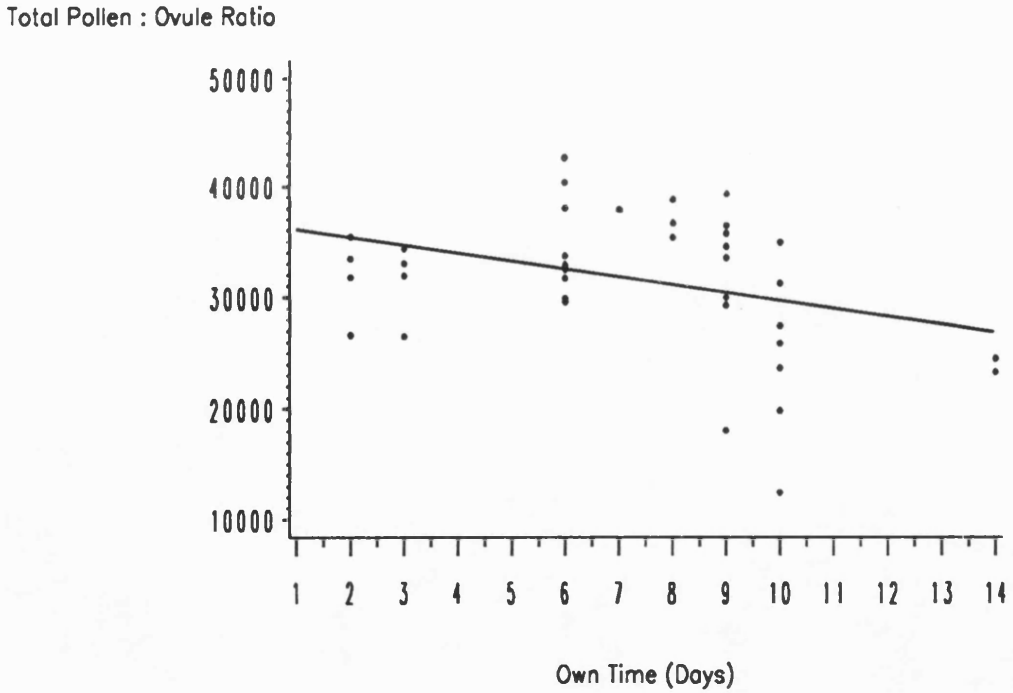
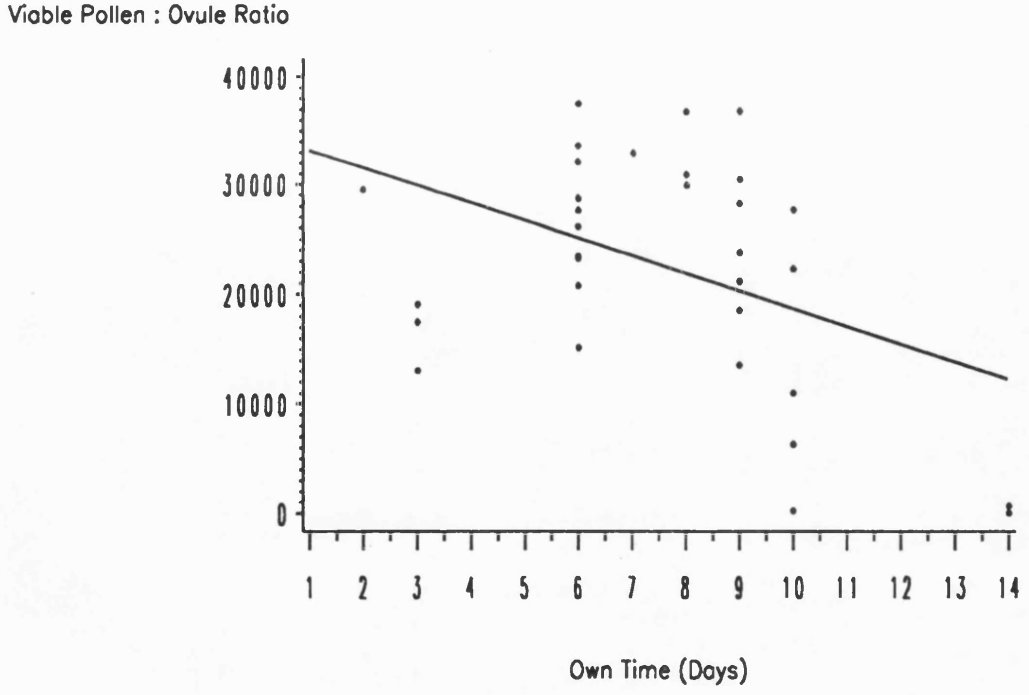


Fig. 3·16 – A Plot Of Variation In Viable Pollen : Ovule Ratio With Time For Greenhouse Plant 4



plants. However, genetic, age, size and time effects could still have been present.

The data suggest significant differences between plants in their overall allocation strategies to gamete production. That is, there are genetic effects in allocation strategy.

For four of the plants, the number of female gametes produced decreased through the flowering season. The rate of decrease was not significantly different between these plants. That is, plants are adopting a similar strategy in the way they allocate resources to female function through the season.

From this result, it might be expected that both vratio and tratio would show a significant increase through the plants' flowering season, unless total and/or viable pollen also show a significant decrease through the flowering season. For plants 1 and 3, therefore, a significant increase in both tratio and vratio would be expected. For plant 2, a significant increase in vratio would be expected. These predictions are not met. This anomaly can be explained by the differences in sample sizes between measured variable within plants. Ovule number was counted in every flower collected. However, it was not always possible to obtain estimates of pollen number and/or viability of pollen grains. In a few cases, anthers had dehisced before the flower could be collected. Some of the preparations for measuring pollen viability were spoiled (e.g. light got into the petri dish, or the

sample was dropped). Values for tratio and vratio could only be calculated where both ovule count and the relevant pollen count were available, not for all values of ovule number. If all data values were available for tratio and vratio, it is probable that a significant regression would have been found. The data from plant 4, which was the largest data set, provide support for this. The plant produced 41 flowers in all. Although some samples were lost, significant regressions were found for all variables. Leaving apart plant 5, where no significant regressions on time were revealed for any of the variables, the number of female gametes showed a significant decrease through the flowering season. Generally, the number of male gametes showed a similar although not significant trend, the exception being plant 1, for which the regression coefficient for both viable and total pollen against time was positive. These positive relationships were not significant, whereas those for female gametes were. It would thus appear that total allocation to gamete production decreased with time. For plants 2-4, since both tratio and vratio also decreased, the number of total and viable male gametes must have been decreasing at a more rapid rate than the number of female gametes. The plants became more female as the flowering season progressed. This was not the case in plant 1 or plant 4.

The general conclusions that can be drawn from these data are as follows:

i) Allocation to female gamete production decreased with time in plants 1-4.

ii) Allocation to male gamete production definitely decreased with time in plant 4 and probably decreased with time in plants 2 and 3, although confirmation of this cannot be provided because of statistical artefacts. Allocation to male gamete production may have increased with time in plant 1, although, again, confirmation of this cannot be provided.

iii) Total allocation to gamete production decreased with time in plants 1-4.

iv) P/O definitely decreased with time in plant 4. Because of the statistical artefacts mentioned in point ii), it is not clear whether a similar change occurred in plants 2 and 3. However, this is likely. In plant 1, there may have been an increase in P/O, but this cannot be confirmed for the reasons given in point ii).

v) There were no significant changes to female gamete production, male gamete production, total gamete production or P/O with time in plant 5.

vi) Thus, overall, in 3 of the 5 plants, there is evidence that total allocation decreases with time, and that this is associated with a move towards femaleness as measured by P/O. In one of the

plants, there is evidence that a decrease in total allocation to gamete production is associated with a move towards maleness. There was no change with time either in total or relative allocation to gametes in one of the 5 plants.

c) An experiment to determine the effect of competition on the gender of *R. bulbosus*

i) Methods

A randomized block design involving six blocks, ten clone types, and two treatments, was used. Each block contained one individual per clone per treatment. Plants were randomly distributed within the blocks at a spacing of 0.5 m between individuals. A sacrificial row of plants was placed around the whole experimental area. Experimental plants were planted on three successive days at the beginning of November, 1986. The experiment took place in the open at the University field station. The treatments used were:

i) control plants grown on their own;
ii) competed plants (see Section 3.2.1 g).

Several measurements were made before, during and after flowering.

i) Rosette size of all experimental plants before the 1987 flowering season.
ii) Total pollen and ovule counts for ten flowers from each experimental plant. Initially, a flower was collected from each plant every other day. The ten flowers to be analysed were selected from these. The first and last flowers from each plant were always included in the sample. Eight other flowers from

dates spaced out through the flowering season were also used. Flowers were stored at +4 °C prior to counting.

iii) Total numbers of flowers produced by each plant.

iv) Duration of the flowering season of each plant, measured in days.

v) Corm size at the end of the 1987 flowering season. This involved destructive sampling and consequently was recorded for block 1 only. The rest of the experimental plot was left intact in case it was required again.

Several calculations were performed on the data:

i) Pollen:ovule ratios (P/O) were calculated for each flower examined.

ii) Lloyd's (1979, 1980) measure of functional gender (G) was calculated for each flower examined.

iii) Total allocation to gametes per flower (T_f) was calculated using the equation

$$T_f = g_i + a_i E$$

where g_i is the total number of ovules in a flower, a_i is the total pollen count per flower, and E is the equivalence factor which equates pollen and ovule counts. In this case

$$E = \frac{\text{total ovule count in the population of experimental plants}}{\text{total pollen count in the population of experimental plants}}$$

iv) Total allocation to gametes per plant (T_P) was estimated by multiplying mean T_f per plant by the total number of flowers produced by the plant during its flowering season.

v) The sum of the total pollen counts for the ten flowers from each plant was divided by the sum of the ovule counts from the same ten flowers. This gave a different measure of mean P/O referred to as 'M'.

vi) Log_{10} of the total pollen count, P/O and rosette size were calculated to transform the data to a normal distribution.

vii) Means of ovule count per flower, log_{10} pollen count per flower, G per flower, T_f and log_{10} P/O per flower were calculated for each plant.

A number of statistical analyses were performed on the data. Two statistical packages were used:

i) MINITAB 5.1.3 on the GEC machine;

ii) GENSTAT, version IV at South Western University Regional Computer Centre (SWURCC).

GENSTAT was used to carry out a two-way analysis of variance (ANOVA) on:

i) mean log_{10} pollen count per flower per plant;

ii) mean ovule count per flower per plant;

iii) mean log_{10} P/O per flower per plant;

iv) M per plant;

v) mean G per flower per plant;

vi) mean T_f per flower per plant;

vii) T_p per plant;

viii) number of flowers produced by each plant in its flowering season;

ix) duration of the flowering season of each plant;

x) $\log_{10}$ rosette size before flowering for each plant.

Unplanned comparisons were carried out between the mean values per clone using the minimum significant difference (MSD) technique (Sokal and Rohlf, 1981).

GENSTAT was also used to perform an analysis of covariance (ANCOVA), using the covariate 'time from the beginning of the plant's own flowering season' ('own' time) measured in days on:

i) $\log_{10}$ total pollen count per flower per plant;

ii) ovule count per flower per plant;

iii) $\log_{10}$ P/O per flower per plant.

Data from all blocks and treatments were pooled separately for each clone. Regression analyses of:

i) total pollen count per flower against time;

ii) ovule count per flower against time;

iii) P/O per flower against time;

were then carried out for each clone using GENSTAT. An ANOVA was performed on the regression coefficients to see if there were any differences between clones. Unplanned comparisons were performed

between regression coefficients for the different clones using the T^1 method (Sokal and Rohlf, 1981).

Using the MINITAB statistical package, rosette size was regressed against:

i) mean ovule count per flower per plant;

ii) mean pollen count per flower per plant;

iii) mean P/O per flower per plant;

iv) mean T_f per flower per plant;

v) T_p per plant;

vi) mean G per flower per plant;

vii) number of flowers produced by each plant in its flowering season;

viii) duration of the flowering season of each plant.

These analyses were performed on all data pooled across block, clone and treatment.

MINITAB was also used to regress corm size against:

i) mean ovule count per flower per plant;

ii) mean pollen count per flower per plant;

iii) mean P/O per flower per plant;

iv) mean T_f per flower per plant;

v) T_p per plant;

vi) number of flowers produced by each plant in its flowering season;

vii) duration of the flowering season of each plant.

These analyses were performed on data from block 1 only. Within the block, data were pooled across clones and treatments.

ii) Results

The ANOVA showed no significant difference ($p > 0.05$) between blocks for any of the variables. There was also no significant treatment effect or clone by treatment interactive effect for any of the variables ($p > 0.05$) for all measured variables. These results are summarized in tables 3.7 - 3.10.

The results indicate that treatment has no effect on overall allocation to reproduction or on allocation pattern to the two sex functions. However, it would appear that the clones are adopting different strategies in both overall allocation and in the pattern of allocation to reproduction.

Although an overall significant difference was found between clones for mean $\log_{10}$ total pollen count per flower, this difference was not detected when an unplanned comparison between the means was performed.

In a situation such as this, when two statistical tests give conflicting results, it is necessary to decide which result is the most appropriate. On taking statistical advice and reading widely around this subject, it was decided that the results of the ANOVA should be accepted, rather than those of the unplanned comparisons between means.

TABLE 3.7 RESULTS OF THE ANALYSIS OF VARIANCE FOR ALL VARIABLES EXCEPT CORM SIZE

* Lloyd (1979, 1980)

VARIABLE	BLOCK EFFECTS			CLONE EFFECTS			TREATMENT EFFECTS			CLONE X TREATMENT EFFECTS		
	F	DEGREES FREEDOM	SIGNI-FICANCE	F	DEGREES FREEDOM	SIGNI-FICANCE	F	DEGREES FREEDOM	SIGNI-FICANCE	F	DEGREES FREEDOM	SIGNI-FICANCE
MEAN OVULE NUMBER	1.03	5,94	p>0.05	56.56	9,94	p<0.001	0.09	1,94	p>0.05	0.87	9,94	p>0.05
MEAN LOG_{10} POLL. COUNT	0.82	5,94	p>0.05	3.04	9,94	p<0.005	0.01	1,94	p>0.05	0.66	9,94	p>0.05
MEAN LOG_{10} RATIO	1.48	5,94	p>0.05	20.77	9,94	p<0.001	0.02	1,94	p>0.05	0.72	9,94	p>0.05
TOTAL POLL. / TOTAL OV.	1.61	5,94	p>0.05	17.24	9,94	p<0.005	2.75	1,94	p>0.05	0.72	9,94	p>0.05
MEAN G*/ FLOWER	1.49	5,94	p>0.05	20.33	9,94	p<0.001	0.004	1,94	p>0.05	0.62	9,94	p>0.05
MEAN TOTAL ALLOCATION/ FLOWER	0.50	5,94	p>0.05	7.27	9,94	p<0.005	0.02	1,94	p>0.05	0.61	9,94	p>0.05
TOTAL ALLOCATION/ PLANT	2.06	5,94	p>0.05	12.63	9,94	p<0.001	2.59	1,94	p>0.05	1.72	9,94	p>0.05
NUMBER OF FLOWERS PRODUCED	2.15	5,95	p>0.05	11.93	9,95	p<0.001	2.33	1,95	p>0.05	1.63	9,95	p>0.05
DURATION OF FLOWERING	0.28	5,95	p>0.05	5.14	9,95	p<0.001	1.09	1,95	p>0.05	1.60	9,95	p>0.05
LOG_{10} ROSETTE SIZE	1.86	5,95	p>0.05	10.14	9,95	p<0.001	0.31	1,95	p>0.05	0.38	9,95	p>0.05

TABLE 3.8 ANOVA TABLE OF MEANS BY CLONE FOR ALL VARIABLES

CLONE	MEAN OVULE COUNT	LOG_{10} MEAN POLLEN COUNT	LOG_{10} MEAN RATIO	LOG_{10} ROSETTE SIZE	NUMBER OF FLOWERS PRODUCED	DURATION OF FLOWERING	TOTAL POLLEN / TOTAL OVULE	TOTAL ALLOCATION PER FLOWER	TOTAL ALLOCATION PER PLANT	MEAN G* PER PLANT
1	ac 42.26	a 5.8049	ade 4.1994	ad 2.084	ad 178.7	ab 46.75	ad 15338	abcd 87.15	ab 15798	ac .4946
2	a 45.46	a 5.7454	c 4.0911	ad 2.040	ac 144.4	a 47.83	b 11046	abcd 85.64	ab 12573	a .5545
3	b 29.64	a 5.8319	b 4.7348	a 2.221	ac 143.2	a 50.92	c 22792	def 77.54	ab 11047	b .3979
4	ab 36.49	a 5.7799	ad 4.2259	c 1.286	bc 89.9	b 35.00	ad 16130	bde 77.99	ab 6987	ab .4795
5	a 44.77	a 5.8370	ae 4.1925	ad 2.082	bc 79.1	ab 40.67	ad 15442	ac 92.82	ab 7271	ac .4983
6	a 44.42	a 5.8175	ae 4.1812	ad 2.068	a 201.0	a 47.67	ab 13798	ce 88.49	a 17892	ac .5044
7	bc 33.34	a 5.7883	d 4.2790	cd 1.735	bcd 117.2	ab 41.17	cd 19043	bde 76.48	ab 8944	cb .4495
8	ab 36.40	a 5.7615	ade 4.2075	bc 1.519	b 61.0	b 34.92	ab 13724	bde 77.19	b 4825	ab .4801
9	bc 34.62	a 5.7504	ad 4.2331	abd 1.780	bc 81.4	ab 41.58	ad 17638	bf 75.02	ab 6350	ab .4793
10	a 45.34	a 5.7746	ce 4.1330	abd 1.893	ac 143.9	ab 46.33	ab 13802	ade 88.19	ab 12833	ac .5317

* Lloyd (1979, 1980) Superscripts indicate significant differences between clones found using the MSD method (Sokal and Rohlf, 1981)

TABLE 3.9/I ANOVA TABLE OF MEANS BY CLONE BY TREATMENT FOR ALL VARIABLES

CLONE	TREAT-MENT	MEAN OVULE COUNT	LOG_{10} MEAN POLLEN COUNT	LOG_{10} MEAN RATIO	LOG_{10} ROSETTE SIZE	NUMBER OF FLOWERS PRODUCED
1	1	60.33	5.8154	4.1985	2.104	212.0
	2	53.33	5.7943	4.2002	2.063	145.3
2	1	53.83	5.7412	4.0848	2.056	150.5
	2	56.00	5.7497	4.0974	2.023	138.3
3	1	32.33	5.8472	4.3825	2.232	146.2
	2	31.33	5.8167	4.3672	2.209	140.3
4	1	47.17	5.7596	4.2092	1.289	93.3
	2	50.86	5.8003	4.2426	1.284	86.5
5	1	59.50	5.8290	4.1933	2.084	84.0
	2	61.50	5.8449	4.1917	2.081	74.2
6	1	56.00	5.7932	4.1617	2.022	189.2
	2	54.83	5.8418	4.2007	2.114	212.8
7	1	43.83	5.8076	4.2964	1.834	142.8
	2	45.83	5.7689	4.2616	1.635	91.5
8	1	42.00	5.7566	4.1994	1.577	65.3
	2	41.52	5.7663	4.2156	1.460	56.7
9	1	32.17	5.7594	4.2594	1.692	56.7
	2	49.83	5.7414	4.2068	1.868	106.2
10	1	64.83	5.7886	4.1406	1.979	164.2
	2	58.33	5.7007	4.1254	1.807	123.7

TABLE 3.9/II ANOVA TABLE OF MEANS BY CLONE BY TREATMENT FOR ALL VARIABLES

CLONE	TREAT-MENT	DURATION OF FLOWERING	TOTAL POLLEN / TOTAL OVULE	TOTAL ALLOCA-TION PER FLOWER	TOTAL ALLOCA-TION PER PLANT	MEAN G* PER PLANT
1	1	46.00	13929	88.64	19151	.495
	2	47.50	16746	85.65	12444	.4941
2	1	53.83	10221	85.72	13281	.558
	2	41.83	11871	85.56	11865	.5511
3	1	50.00	22926	78.67	11415	.3933
	2	51.83	22658	76.40	10680	.4024
4	1	37.17	14831	75.44	6942	.4888
	2	32.83	17429	80.55	7033	.4702
5	1	37.33	15165	90.01	7490	.4976
	2	44.00	15719	95.63	7052	.4989
6	1	49.00	13641	85.91	16351	.5154
	2	46.33	13955	91.07	19433	.4933
7	1	42.67	19397	78.39	11201	.4397
	2	39.67	18689	74.56	6688	.4593
8	1	38.67	12656	77.44	5314	.4955
	2	31.17	14792	76.93	4336	.4846
9	1	37.67	17868	75.18	4676	.4684
	2	45.50	17408	74.86	8024	.4903
10	1	48.50	13946	89.95	14878	.5275
	2	44.17	13655	86.44	10788	.5360

* Lloyd (1979, 1980)

TABLE 3.10 ANOVA TABLE OF MEANS BY TREATMENT FOR ALL VARIABLES

TREAT-MENT	MEAN OVULE COUNT	LOG_{10} MEAN POLLEN COUNT	LOG_{10} MEAN RATIO	LOG_{10} ROSETTE SIZE	NUMBER OF FLOWERS PRODUCED	DURATION OF FLOWERING	TOTAL POLLEN / TOTAL OVULE	TOTAL ALLOCATION PER FLOWER	TOTAL ALLOCATION PER PLANT	MEAN G* PER PLANT
1	49.20	5.7898	4.2126	1.887	130.4	44.08	15458	82.53	11070	.4878
2	50.34	5.7885	4.2109	1.855	117.5	42.48	16292	82.77	9834	.4882

* Lloyd (1979, 1980)

[N.B. To find possible differences between pairs of values where it was previously unplanned which pairs of values should be compared, an overall ANOVA should first be carried out. If an overall significant result is found then an unplanned comparison should be used to find differences between particular pairs of values. If a t-test was used to examine the difference between each pair of values, the possibility of producing a type I error (rejection of a true null hypothesis) would increase with every t-test performed. Using unplanned multiple comparisons is a conservative approach which employs an experimentwise rather than testwise type I error rate. If only unplanned comparisons are employed, the chance of making a type II error (acceptance of a false null hypothesis) would be increased. This would certainly have been the case for mean pollen count in this experiment.]

The next step in the analysis of this data was to carry out an ANCOVA on the variables mean $\log_{10}$ pollen count, mean ovule count, and mean $\log_{10}$ P/O, using 'own' time as the covariate. Two prerequisites must be met by the data before an ANCOVA can be performed (Sokal and Rohlf, 1981). Firstly there must be a significant regression of the variable on the co-variate. Secondly, the slopes of the different regression lines should not be significantly different. Although the first condition was met for all clones for the variables ovule number and total pollen count, and for most clones for the variable P/O (see table 3.3), comparison of the regression coefficients showed significant differences between the slopes of the regression lines for the

different clones (see table 3.4). In light of this, the results from the ANCOVA which involve conclusions about clone effects or clone by treatment effects cannot be considered. However, as regards regressions against 'own' time for results pooled by treatment, both conditions are met (see table 3.15). The ANCOVA did not reveal any significant treatment effects. These results are summarised in tables 3.11 - 3.12.

Time appears to be an important factor in determining allocation to gamete production. This is suggested by the amount of variation caused by 'own' time in the ANCOVA. As has been mentioned, regression analyses of total pollen count per flower and total ovule count per flower against 'own' time show significant negative linear regressions for all clones. This was also shown for P/O with most clones, the exceptions being clones 1, 4 and 6. The results are summarised in table 3.13 and graphically presented in Figs. 3.17 to 3.46. Since both pollen count and ovule number exhibit a significant decrease as the flowering season progresses, it would seem apparent that total allocation to gamete production is also decreasing. That is, plants are becoming less reproductively active in terms of the production of new progeny, although they may still be maturing progeny produced earlier in the season. Comparison of the regression coefficients of pollen, ovule and P/O against 'own' time again shows significant differences between clones. The results of this analysis are summarised in table 3.14. This analysis suggests that production of both pollen and ovules

decreases through the flowering season. The rate at which this decrease occurs varies between clone types. In clones 1, 4 and 6, in which P/O remains fairly constant throughout the flowering season, shown by the lack of a significant regression coefficient, the relative rate of decrease of pollen and ovule production must be fairly similar. For the other clones, where there is a significant negative regression of P/O with time, pollen production is decreasing more rapidly than ovule production. These plants are becoming relatively more female as the flowering season progresses, but they are also becoming less reproductively active.

The significant differences in regression coefficients between plants shows that plants are adopting different strategies in the way in which they allocate resources to male and female gamete production as the flowering season progresses. Some of the most apparent results from the unplanned comparisons between regression coefficients are that the rate at which clone 8 decreases its allocation to male gamete production is significantly lower than the rate of decrease for clones 9 and 10. Clone 8 also decreases its allocation to female gamete production at a significantly lower rate than all other clones. However, the rate at which P/O decreases for clone 8 is not significantly different to any of the other clones except for clone 9, which exhibits a significantly higher rate of decrease than all other clones (including clone 8). As was discussed in the section concerning the ANOVA, unplanned comparisons between means provide

TABLE 3.11 RESULTS FOR THE ANALYSIS OF CO-VARIANCE FOR OVULE NUMBER, LOG_{10} POLLEN COUNT AND LOG_{10} P/O

VARIABLE	BLOCK EFFECTS			TREATMENT EFFECTS			PERCENTAGE VARIATION EXPLAINED BY	
	F	DEGREES FREEDOM	SIG-NIF-ICA-NCE	F	DEGREES FREEDOM	SIG-NIF-ICA-NCE	CO-VARIATE TIME	TREATMENT
MEAN OVULE NUMBER	0.2	5, 1042	>0.05	2.72	1, 1042	>0.05	32.77%	0.09%
LOG_{10} MEAN POLLEN COUNT	0.18	5, 1042	>0.05	2.61	1, 1042	>0.05	38.82%	0.15%
LOG_{10} MEAN P/O	0.28	5, 1042	>0.05	0.85	1, 1042	>0.05	8.58%	0.06%

TABLE 3.12 ANCOVA TABLE OF MEANS BY TREATMENT ADJUSTED FOR THE CO-VARIATE 'OWN' TIME

TREATMENT	OVULE COUNT	LOG_{10} POLLEN COUNT	LOG_{10} RATIO
1	39.54	13.347	9.7007
2	38.97	13.317	9.6844

TABLE 3.13/I RESULTS OF REGRESSION ANALYSES OF POLLEN COUNT, OVULE NUMBER AND P/O AGAINST 'OWN' TIME, CARRIED OUT BY CLONE

		CLONE 1	CLONE 2	CLONE 3	CLONE 4	CLONE 5	CLONE 6	CLONE 7	CLONE 8	CLONE 9	CLONE 10
POLLEN	Regn coeff.	ab -13721	ab -11017	ab -11618	ab -8351	ab -15816	ab -8221	ab -14632	b -7187	a -17153	a -18808
	F	75.15	43.16	58.8	28.62	57.25	60.56	92.91	22.05	107.45	146.39
	t	-8.67	-6.57	-7.67	-5.35	-7.57	-7.78	-9.64	-4.70	-10.37	-12.1
	df	1,109	1,97	1,108	1,106	1,110	1,105	1,112	1,86	1,106	1,111
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Superscripts indicate significant differences between clones found using the T^1 method (Sokal and Rohlf, 1981)

TABLE 3.13/II RESULTS OF REGRESSION ANALYSES OF POLLEN COUNT, OVULE NUMBER AND P/O AGAINST 'OWN' TIME, CARRIED OUT BY CLONE

OVULES

	CLONE 1	CLONE 2	CLONE 3	CLONE 4	CLONE 5	CLONE 6	CLONE 7	CLONE 8	CLONE 9	CLONE 10
Regn coeff.	^{a} -0.634	^{c} -0.367	^{bd} -0.15	^{c} -0.387	^{ac} -0.525	^{c} -0.425	^{c} -0.433	^{b} -0.0708	^{cd} -0.303	^{a} -0.666
F	299.6	88.9	36.01	92.25	206.95	160.69	165.17	10.45	43.67	334.64
t	-17.32	-9.43	-6.00	-9.60	-14.38	-12.68	-12.84	-3.23	-6.61	-18.25
df	1,118	1,118	1,118	1,116	1,118	1,118	1,118	1,113	1,108	1,118
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.005^{F} / 0.01^{t}	<0.001	<0.001

Superscripts indicate significant differences between clones found using the T^{1} method (Sokal and Rohlf, 1981)

TABLE 3.13/III RESULTS OF REGRESSION ANALYSES OF POLLEN COUNT, OVULE NUMBER AND P/O AGAINST 'OWN' TIME, CARRIED OUT BY CLONE

RATIO	CLONE 1	CLONE 2	CLONE 3	CLONE 4	CLONE 5	CLONE 6	CLONE 7	CLONE 8	CLONE 9	CLONE 10
Regn coeff.	a -65.3	a -90.7	a -314	a -50.1	a -142	a -27.8	a -145	a -156	b -876	a -185
F	2.72	6.92	35.9	1.58	13.86	1.23	12.09	16.39	14.56	38.83
t	-1.65	-2.63	-5.99	-1.26	-3.72	-1.11	-3.48	-4.05	-3.82	-6.23
df	1,109	1,97	1,108	1,106	1,110	1,105	1,112	1,86	1,106	1,111
p	>0.05	0.025^F / 0.02^t	<0.001	>0.05	<0.001	>0.05	<0.001	<0.001	<0.001	<0.001

Superscripts indicate significant differences between clones found using the T^1 method (Sokal and Rohlf, 1981)

TABLE 3.14

COMPARISONS OF REGRESSION COEFFICIENT BETWEEN CLONES FOR POLLEN OVULES AND RATIO, PLOTTED AGAINST TIME OF PLANTS' FLOWERING SEASON

VARIABLE	F	DEGREES FREEDOM	P
OVULE	35.60	9,1163	<0.001
POLLEN	6.61	9,1050	<0.001
RATIO	9.30	9,1050	<0.001

TABLE 3.15 SUMMARY OF REGRESSION ANALYSES AGAINST TIME FOR DATA GROUPED BY TREATMENT

	POLLEN COUNT AGAINST TIME		OVULE COUNT AGAINST TIME		P/O AGAINST TIME	
	TREATMENT 1	TREATMENT 2	TREATMENT 1	TREATMENT 2	TREATMENT 1	TREATMENT 2
Regression coefficient	0.0065	0.0063	0.34	0.33	0.0033	0.0030
F	277.66	209.76	206.42	224.65	54.95	49.67
t	-16.68	-14.48	-14.41	-14.99	-7.41	-7.04
df	1,518	1,548	1,588	1,591	1,518	1,548
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

N.B. No significant difference between treatments for regression coefficients ($p > 0.05$)

Fig 3.17 – Variation Of Total Pollen Count With Time For Clone 1 (all blocks and treatments grouped)

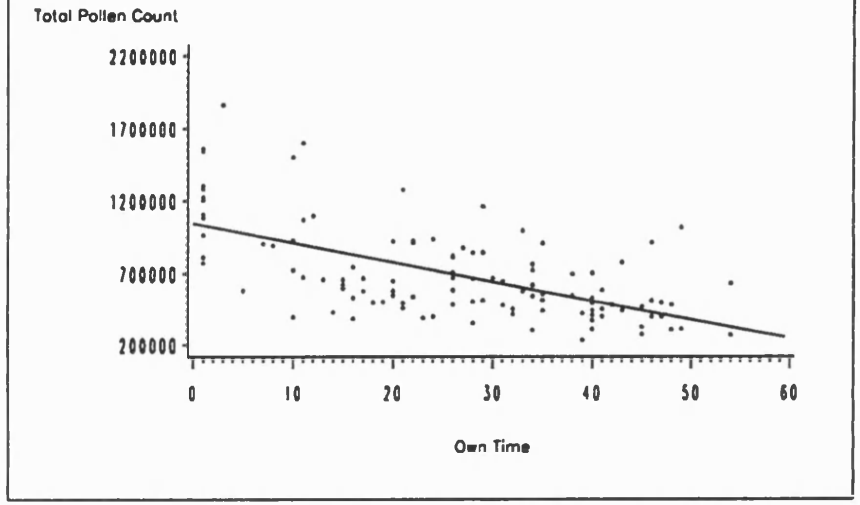


Fig 3.18 – Variation Of Total Pollen Count With Time For Clone 2 (all blocks and treatments grouped)

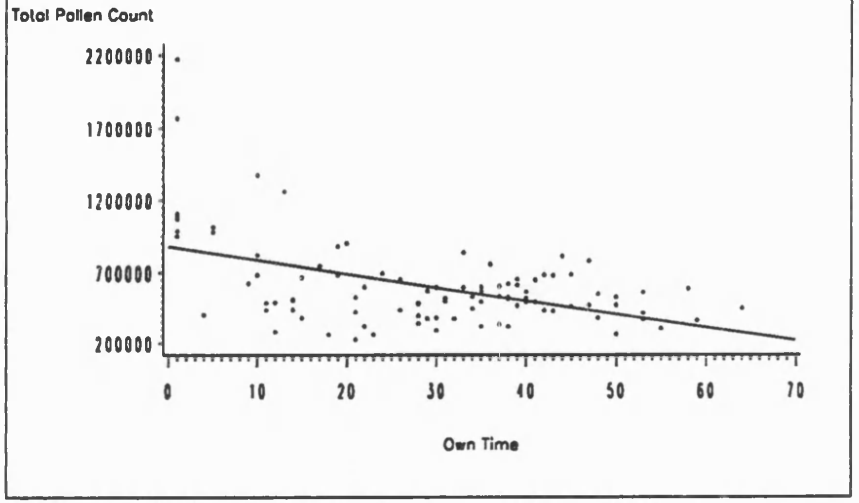


Fig 3.19 – Variation Of Total Pollen Count With Time For Clone 3 (all blocks and treatments grouped)

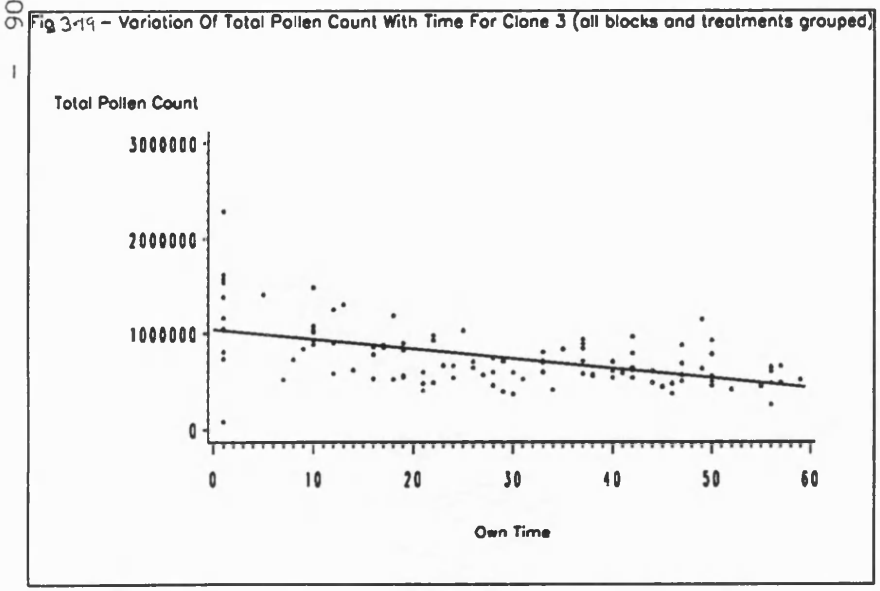


Fig 3.20 – Variation Of Total Pollen Count With Time For Clone 4 (all blocks and treatments grouped)

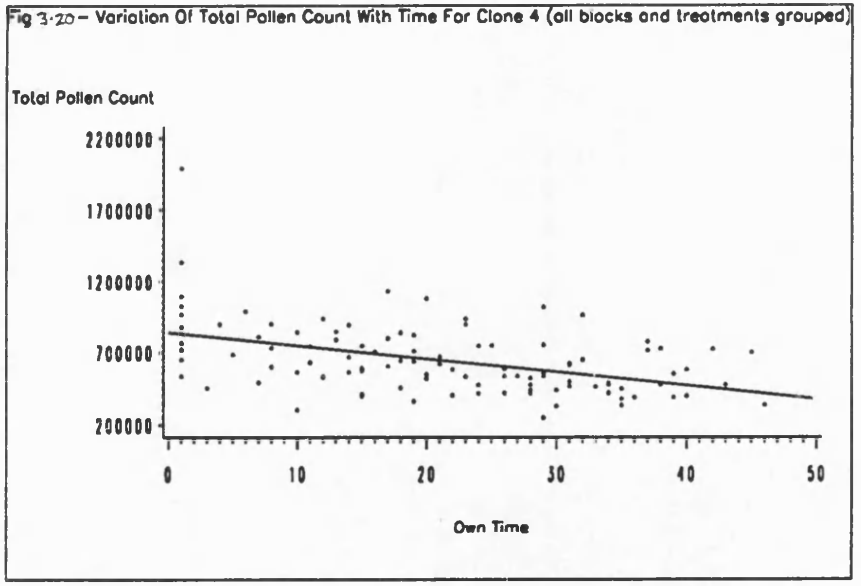


Fig 3·21 – Variation Of Total Pollen Count With Time For Clone 5 (all blocks and treatments grouped)

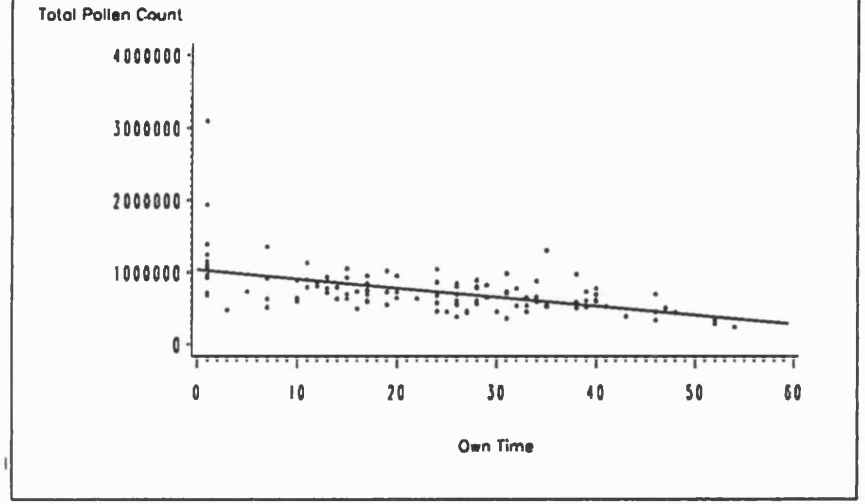


Fig 3·22 – Variation Of Total Pollen Count With Time For Clone 6 (all blocks and treatments grouped)

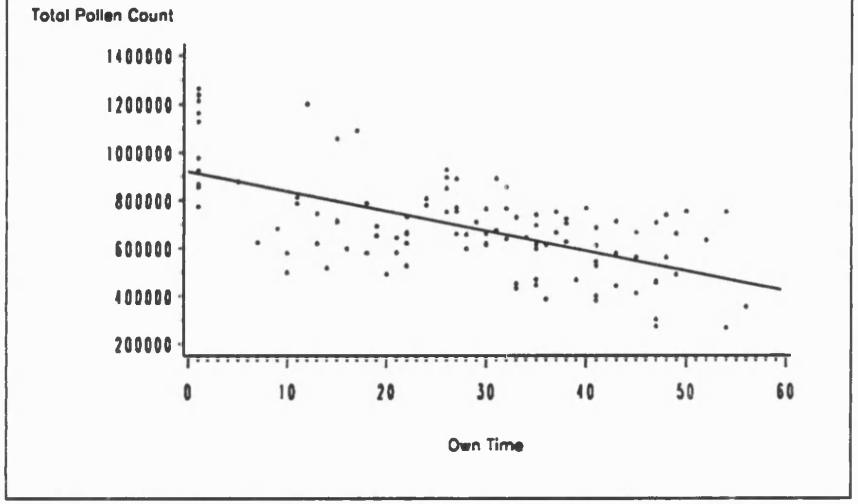


Fig 3·23 – Variation Of Total Pollen Count With Time For Clone 7 (all blocks and treatments grouped)

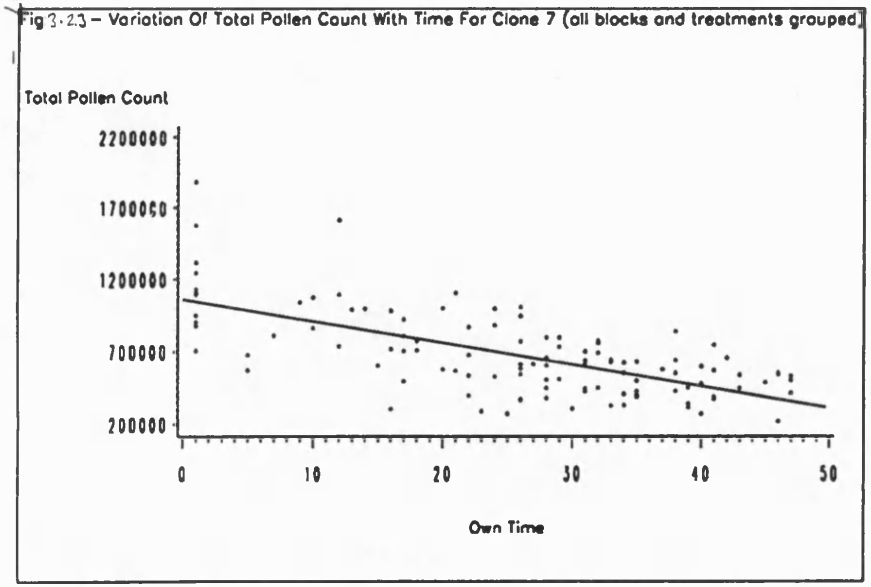


Fig 3·24 – Variation Of Total Pollen Count With Time For Clone 8 (all blocks and treatments grouped)

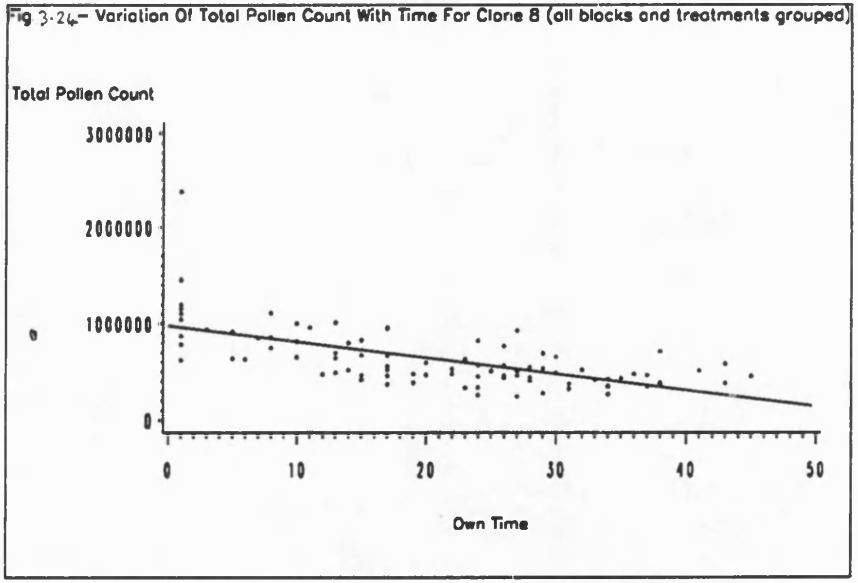


Fig 3-25– Variation Of Total Pollen Count With Time For Clone 9 (all blocks and treatments grouped)

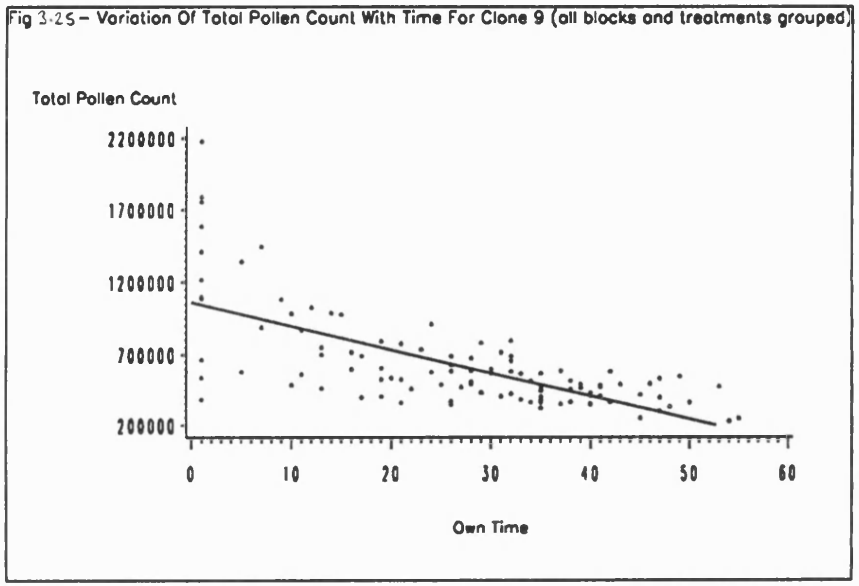


Fig 3-26– Variation Of Total Pollen Count With Time For Clone 10 (all blocks and treatments grouped)

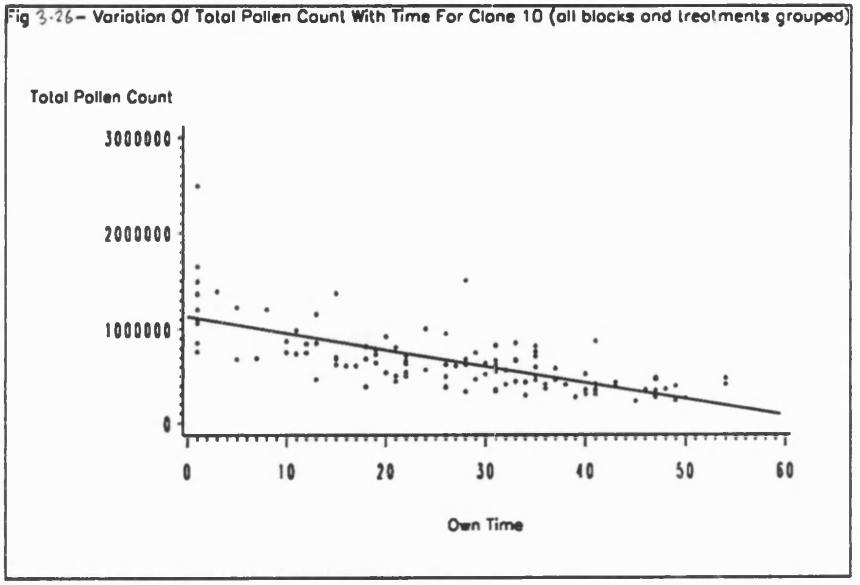


Fig 3.27 – Variation Of Ovule Count With Time For Clone 1 (all blocks and treatments grouped)

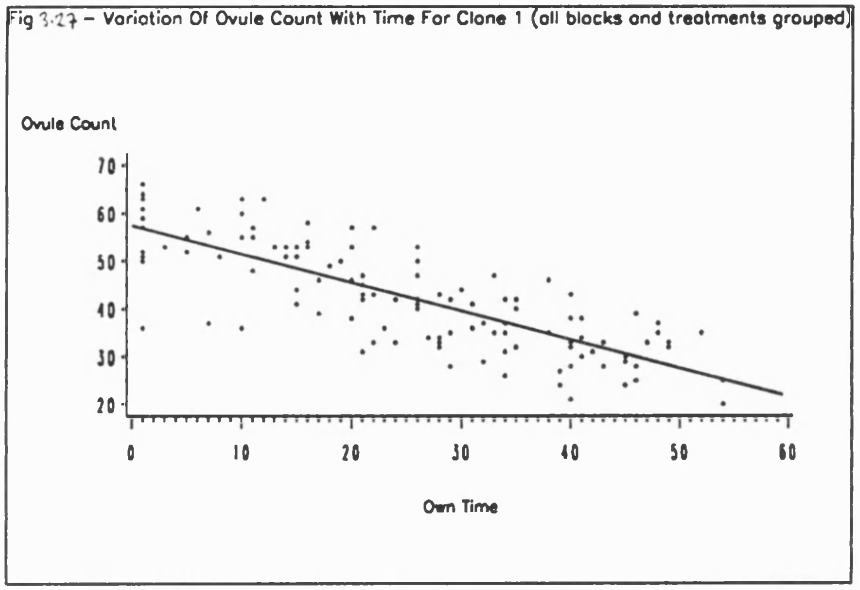


Fig 3.28 – Variation Of Ovule Count With Time For Clone 2 (all blocks and treatments grouped)

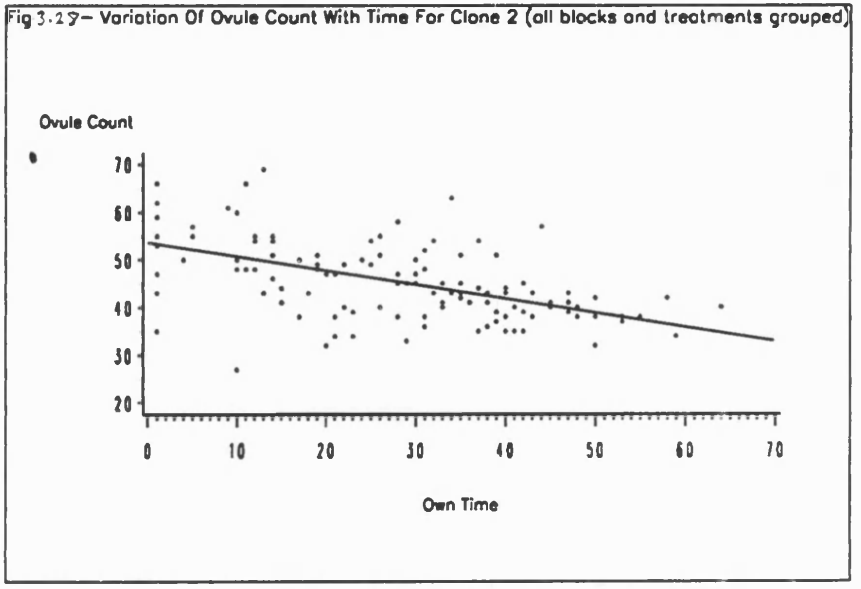


Fig 3.29 – Variation Of Ovule Count With Time For Clone 3 (all blocks and treatments grouped)

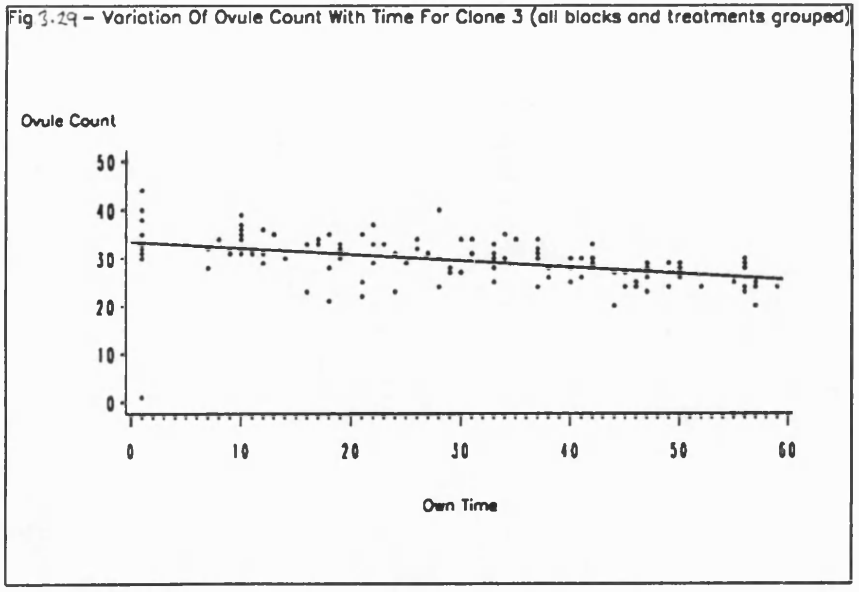


Fig 3.30 – Variation Of Ovule Count With Time For Clone 4 (all blocks and treatments grouped)

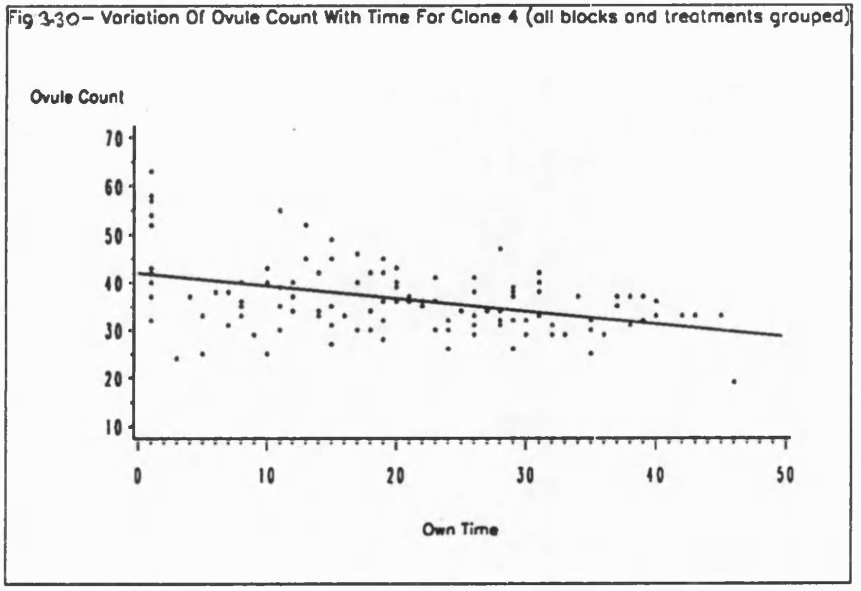


Fig 3·31 – Variation Of Ovule Count With Time For Clone 5 (all blocks and treatments grouped)

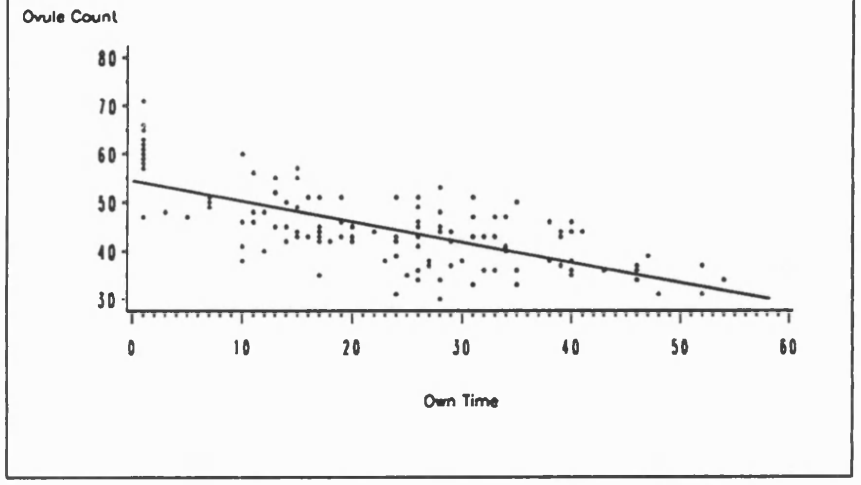


Fig 3·32 – Variation Of Ovule Count With Time For Clone 6 (all blocks and treatments grouped)

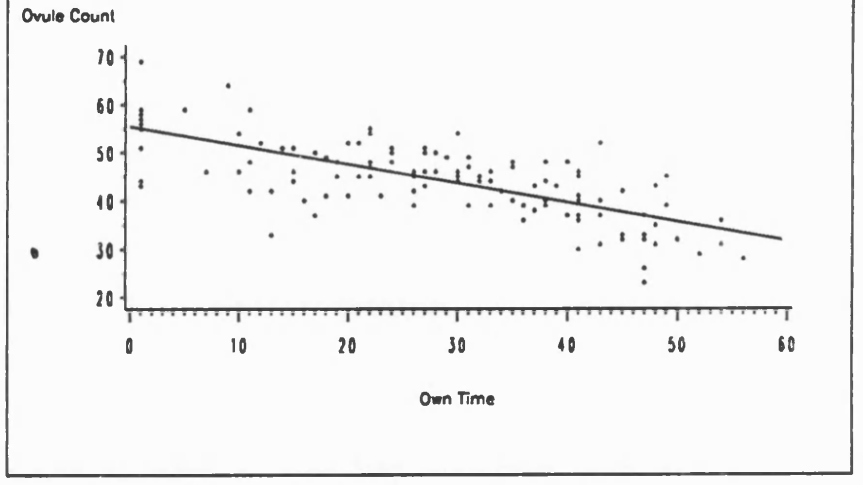


Fig 3·33 – Variation Of Ovule Count With Time For Clone 7 (all blocks and treatments grouped)

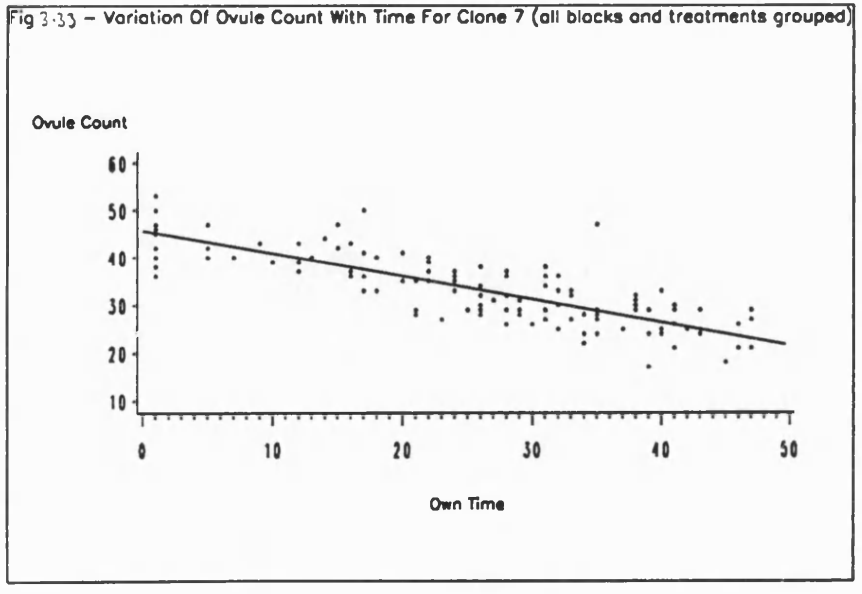


Fig 3·34 – Variation Of Ovule Count With Time For Clone 8 (all blocks and treatments grouped)

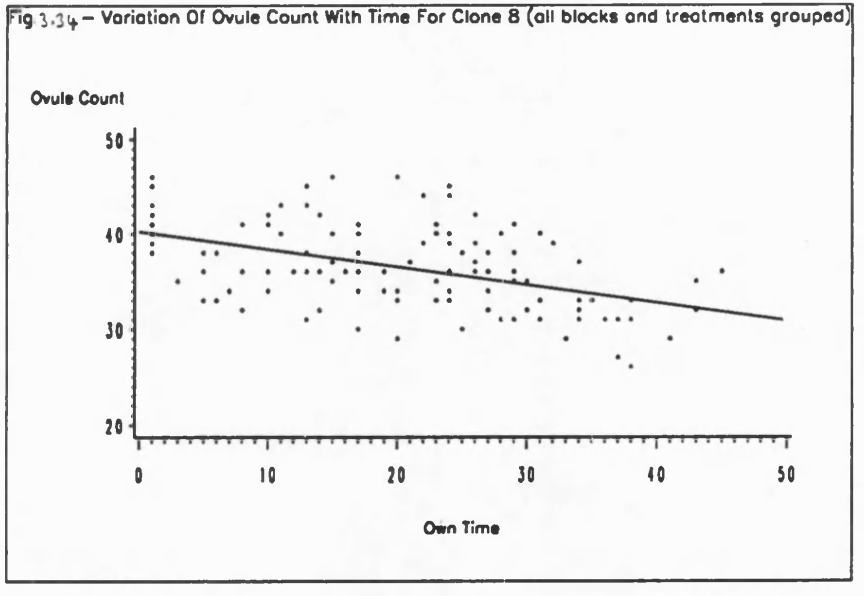


Fig 3·35 – Variation Of Ovule Count With Time For Clone 9 (all blocks and treatments grouped)

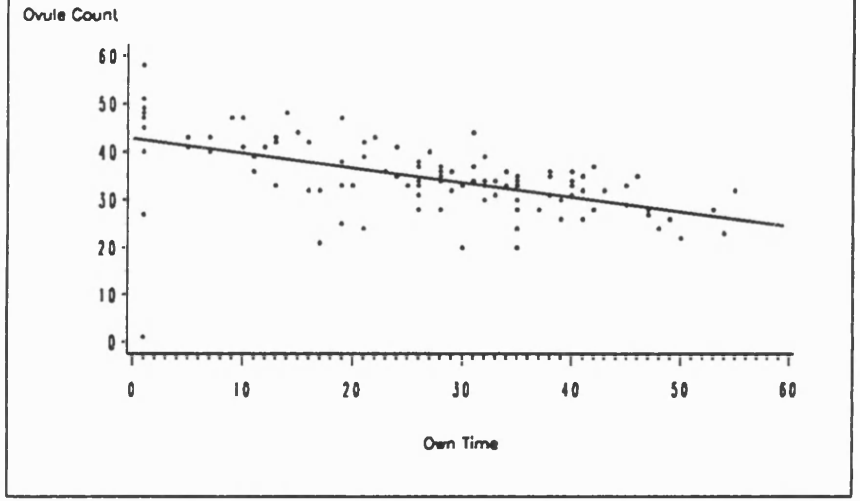


Fig 3·36 – Variation Of Ovule Count With Time For Clone 10 (all blocks and treatments grouped)

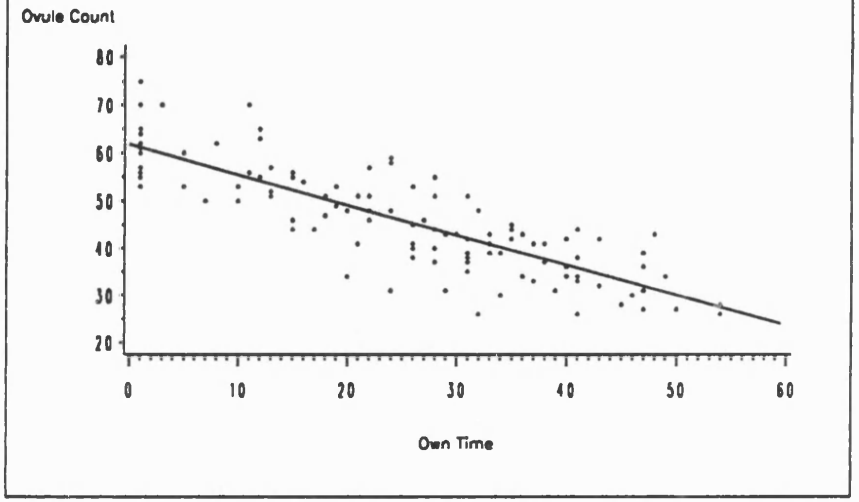


Fig 3.37 – Variation Of Pollen:Ovule Ratio With Time For Clone 1 (all blocks and treatments grouped)

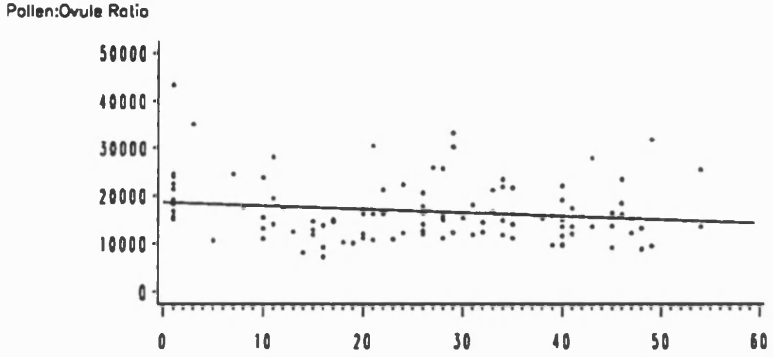


Fig 3.38 – Variation Of Pollen:Ovule Ratio With Time For Clone 2 (all blocks and treatments grouped)

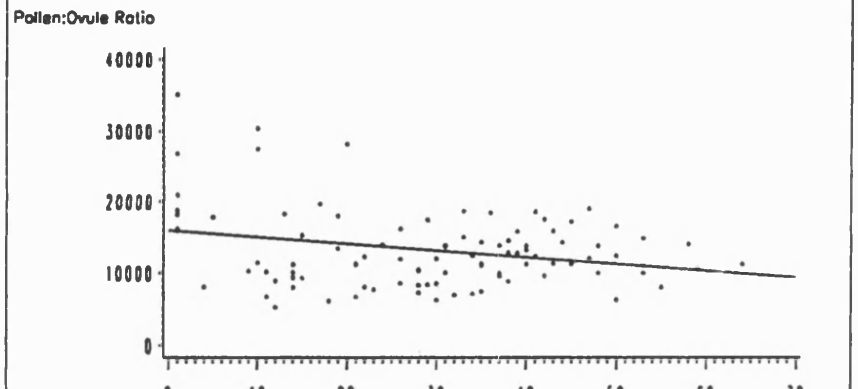


Fig 3.39 – Variation Of Pollen:Ovule Ratio With Time For Clone 3 (all blocks and treatments grouped)

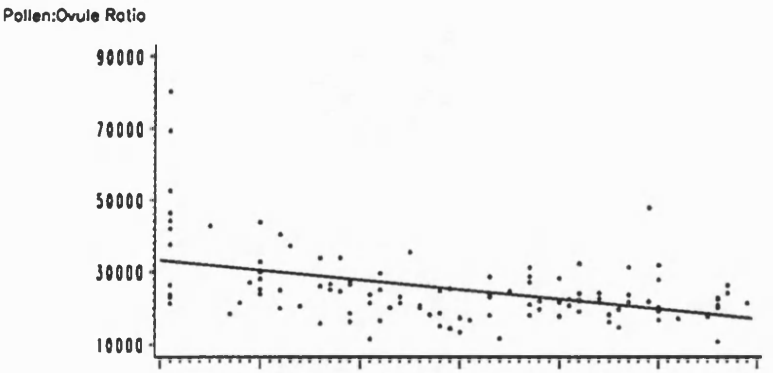


Fig 3.40 – Variation Of Pollen:Ovule Ratio With Time For Clone 4 (all blocks and treatments grouped)

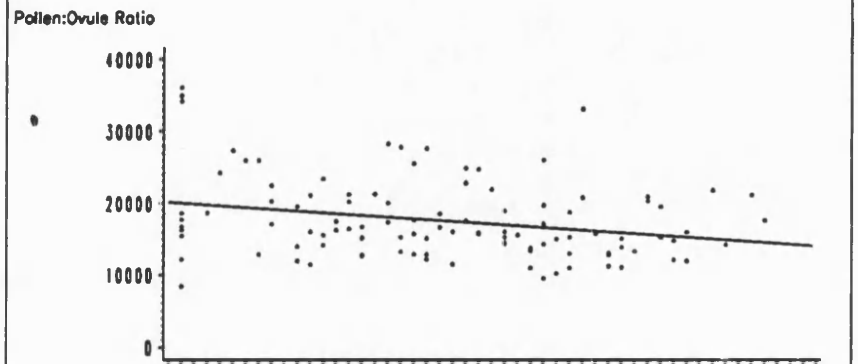


Fig 3.41 – Variation Of Pollen:Ovule Ratio With Time For Clone 5 (all blocks and treatments grouped)

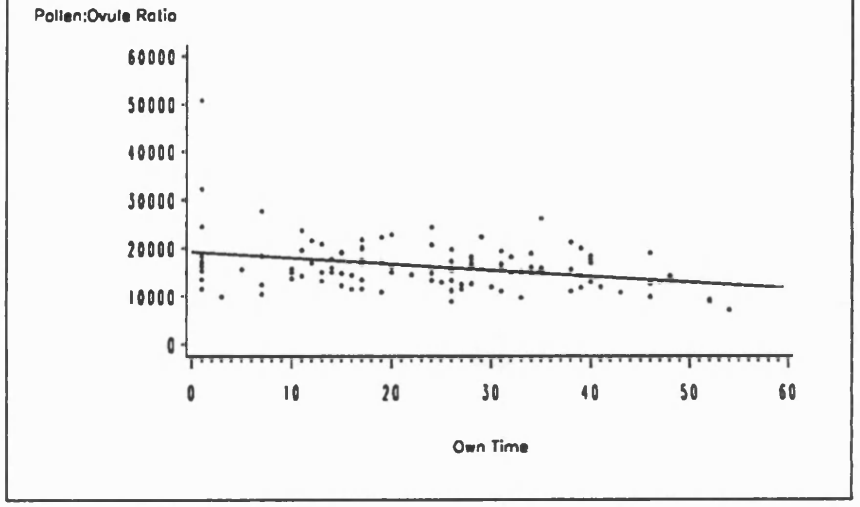


Fig 3.42 – Variation Of Pollen:Ovule Ratio With Time For Clone 6 (all blocks and treatments grouped)

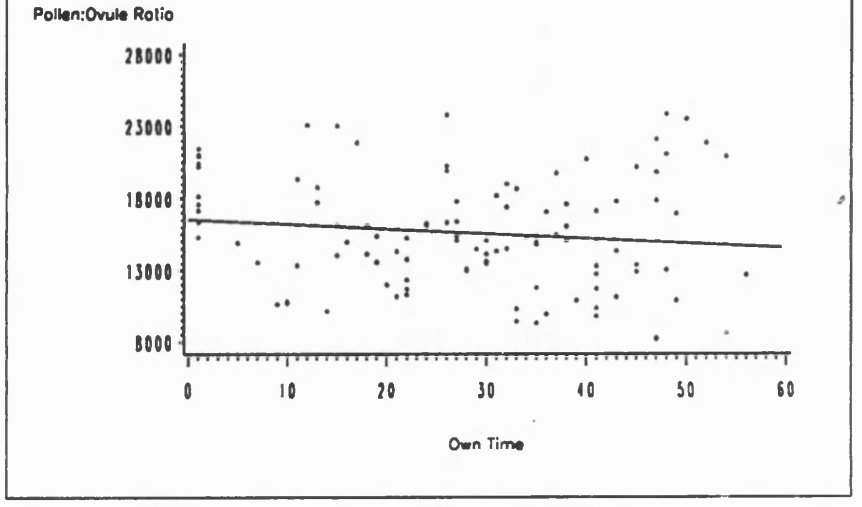


Fig 3.43 – Variation Of Pollen:Ovule Ratio With Time For Clone 7 (all blocks and treatments grouped)

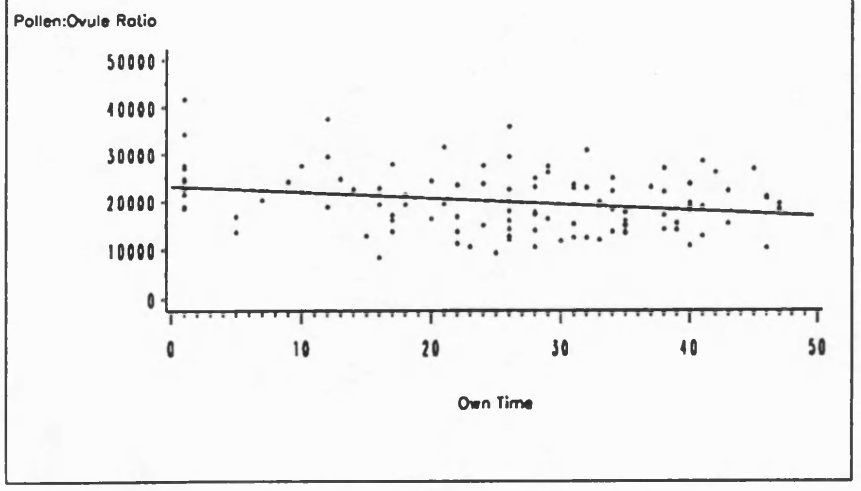


Fig 3.44 – Variation Of Pollen:Ovule Ratio With Time For Clone 8 (all blocks and treatments grouped)

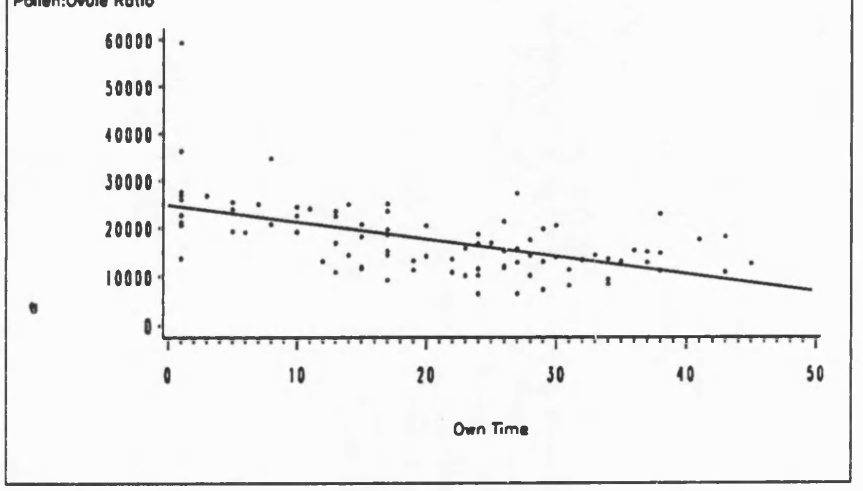


Fig 3.45– Variation Of Pollen:Ovule Ratio With Time For Clone 9 (all blocks and treatments grouped)

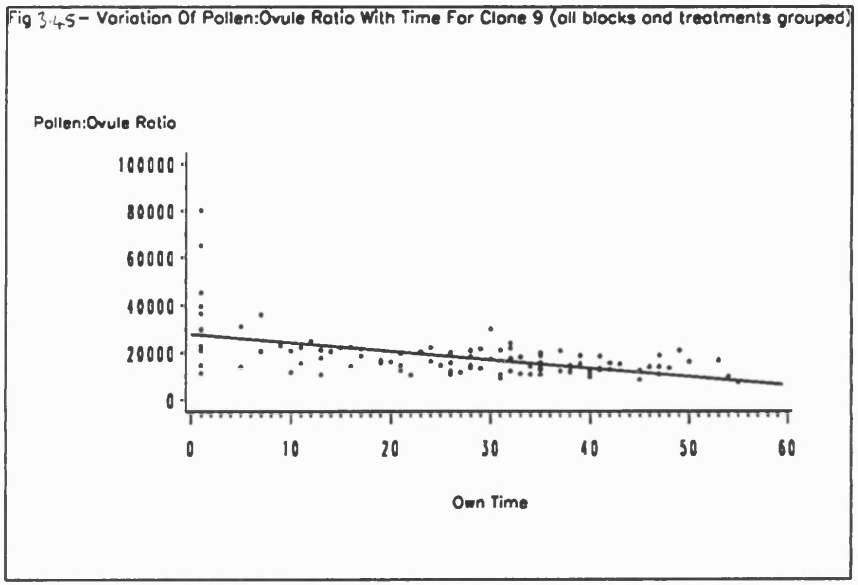
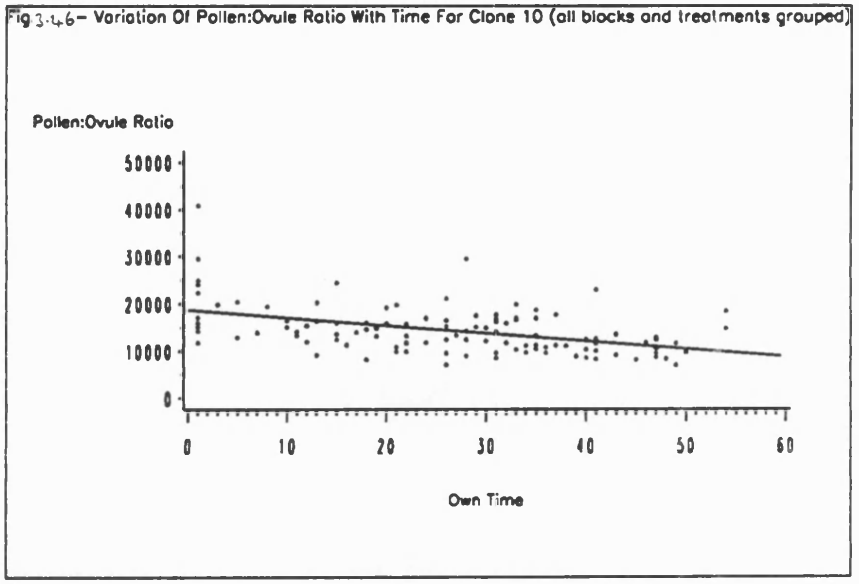


Fig 3.46– Variation Of Pollen:Ovule Ratio With Time For Clone 10 (all blocks and treatments grouped)



a very conservative test. The comparison of regression slopes, beyond finding an overall difference between slopes, is essentially such a series of unplanned comparisons. Being conservative, it might mask real differences between particular pairs of clones, e.g. clone 8 and one or more of the other clones.

Regression analyses against rosette size of the plant before flowering, for the number of flowers produced by a plant during its flowering season, for the length of a plant's flowering season, for the total allocation to reproduction per plant and per flower, and for the mean pollen count per plant, all show significant positive regressions. Results are summarised in table 3.16 and Figs. 3.47 - 3.51. That is, total allocation to reproduction and length of the flowering season are both greater in plants that are large at the start of the flowering season than in plants that are smaller. The absence of a significant regression for the mean ovule count per plant against rosette size indicates that the increase in total reproductive effort is a result of increased pollen and flower production. The increase in number of flowers, together with the per flower effect, generates the overall increase in reproductive effort per plant. No significant regression was found for P/O against rosette size, although a positive relationship might have been expected because of the results obtained when regressing mean pollen count per plant and mean ovule number per plant against rosette size. The lack of a significant regression suggests that there must have been some increase in ovule production with increased rosette

TABLE 3.16 REGRESSION ANALYSIS OF ROSETTE SIZE BEFORE FLOWERING *VERSUS* ALL OTHER VARIABLES

	REGRESSION ANALYSIS ON ROSETTE SIZE *VERSUS*							
	MEAN OVULE COUNT	MEAN POLLEN COUNT	MEAN RATIO	MEAN TOTAL ALLOCATION PER FLOWER	TOTAL ALLOCATION PER PLANT	NUMBER OF FLOWERS PRODUCED	DURATION OF FLOWERING	MEAN G*
Regression coefficient	0.011	265	3.75	0.03	46.8	0.524	0.612	8×10^{-6}
Intercept value	38.1	644219	17339	79.5	5556	68.8	36.8	0.489
F	2.52	4.47	0.40	7.32	72.78	81.06	38.15	0.018
t	1.59	2.11	0.64	2.71	8.53	9.00	6.18	-0.13
Degrees of freedom	1,117	1,117	1,117	1,117	1,117	1,118	1,118	1,117
p	>0.05	<0.05	>0.05	<0.01	<0.01	<0.01	<0.01	>0.05

* Lloyd (1979, 1980)

Fig. 3-47- Relationship Between Rosette Size Before Flowering And Number Of Flowers Produced By A Plant

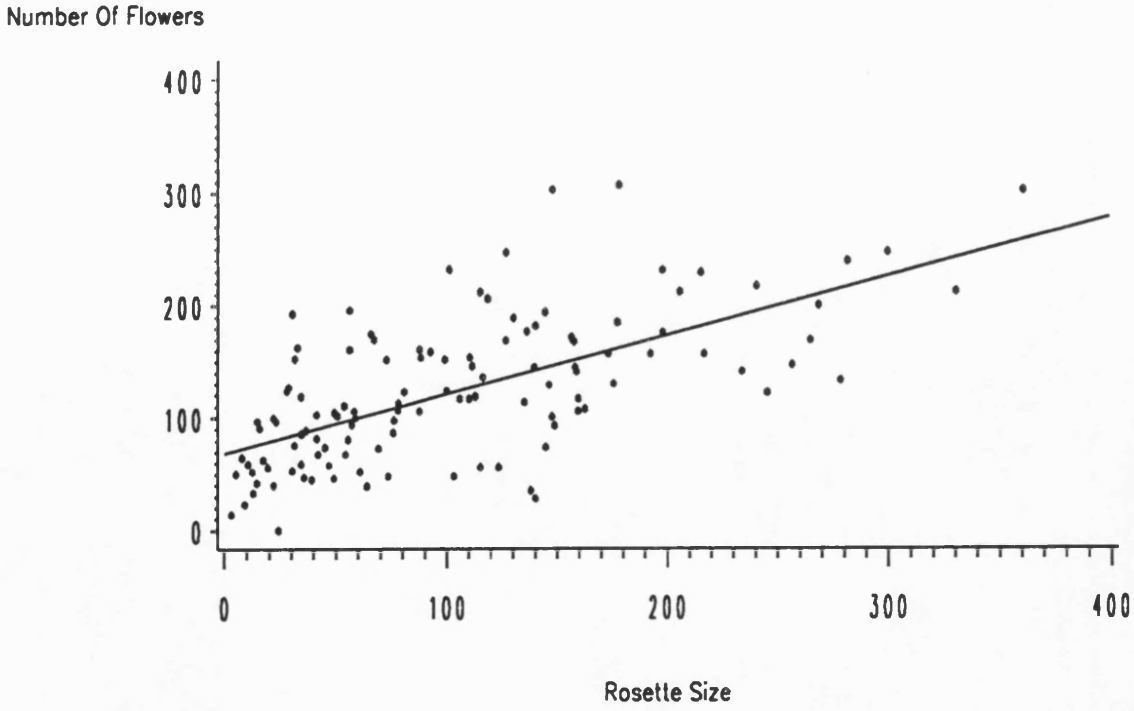


Fig. 3.48– Relationship Between Rosette Size Before Flowering And Duration Of Flowering For A Plant

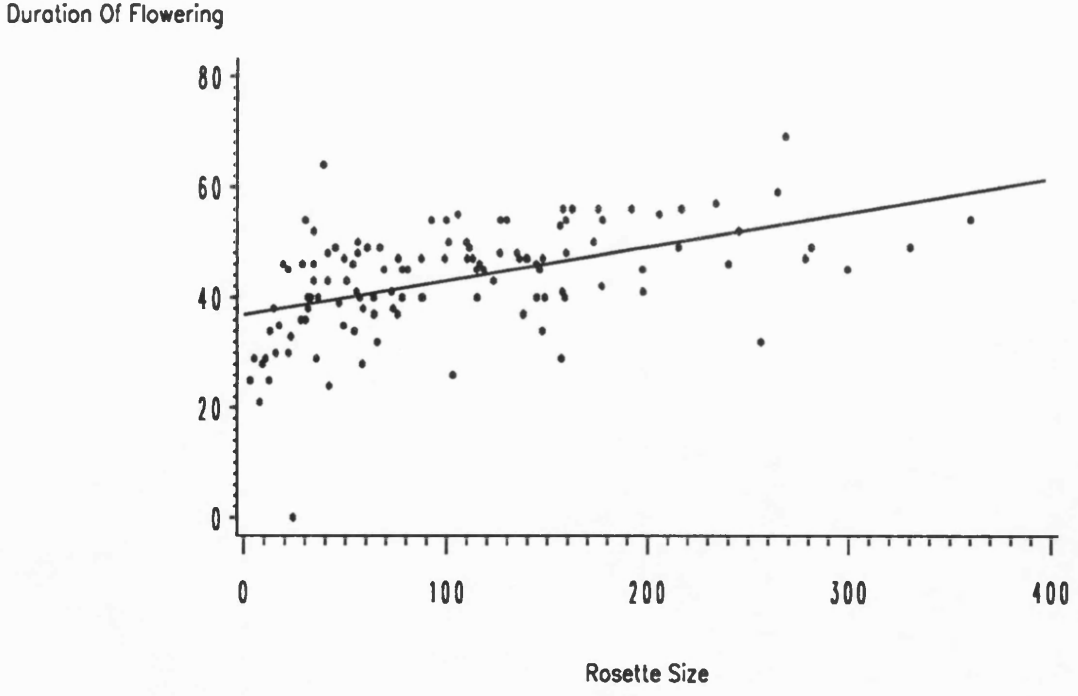


Fig. 3·49 – Relationship Between Rosette Size Before Flowering And Total Allocation To Reproduction/Plant

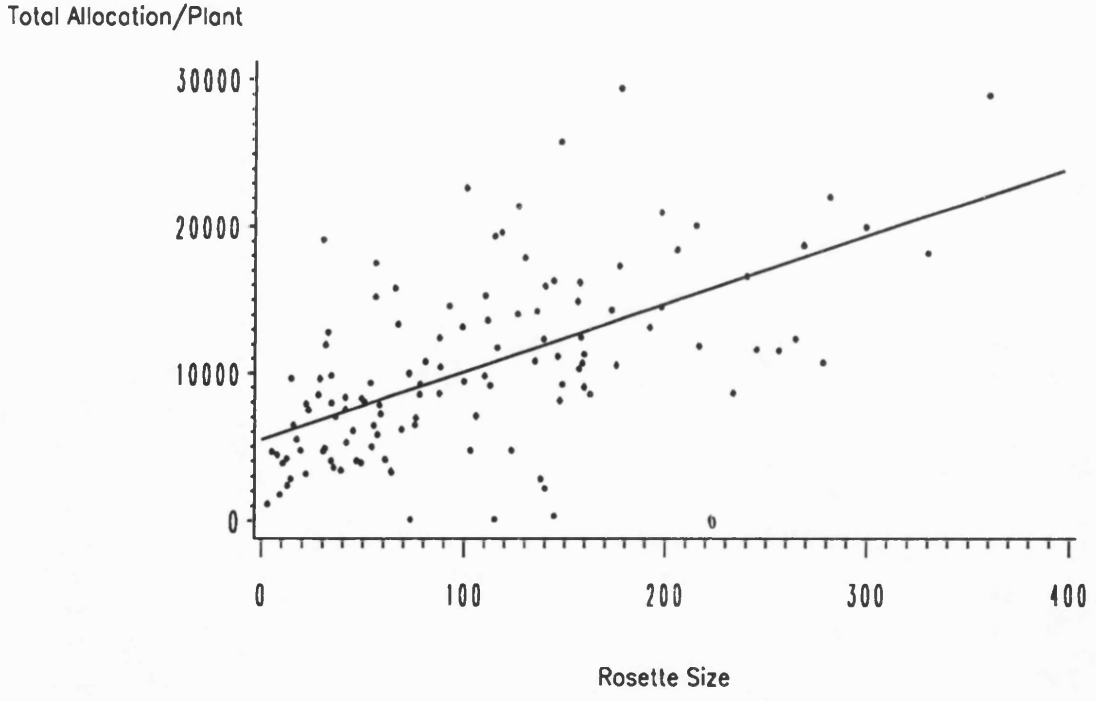


Fig. 3-50– Relationship Between Rosette Size Before Flowering And Mean Total Pollen Count/Flower

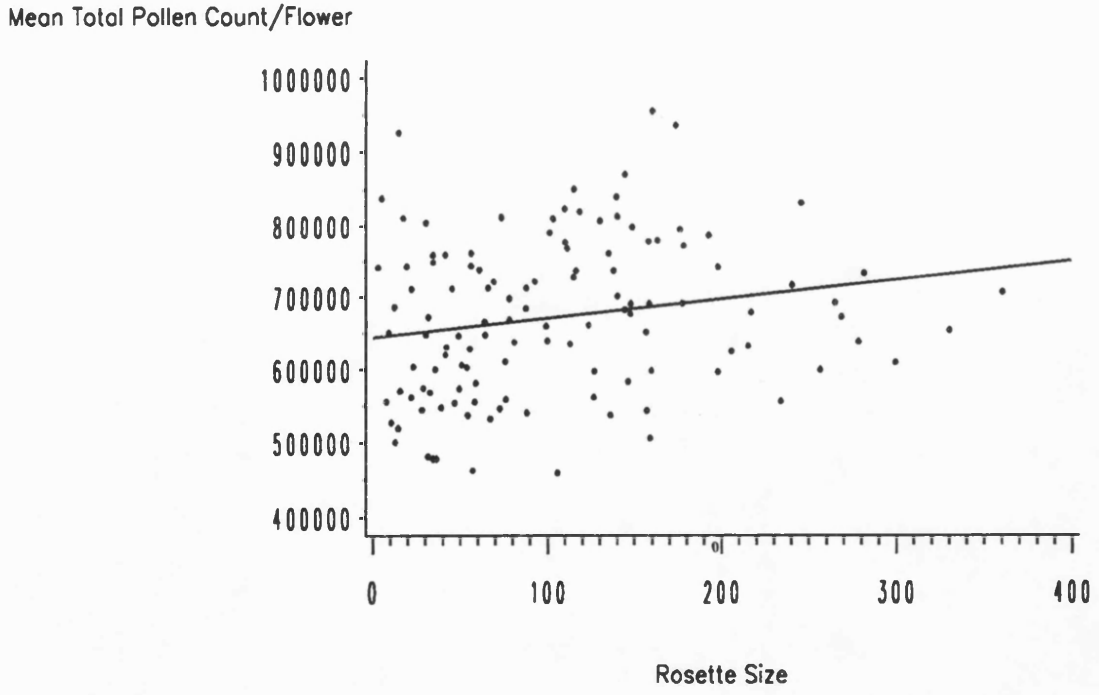
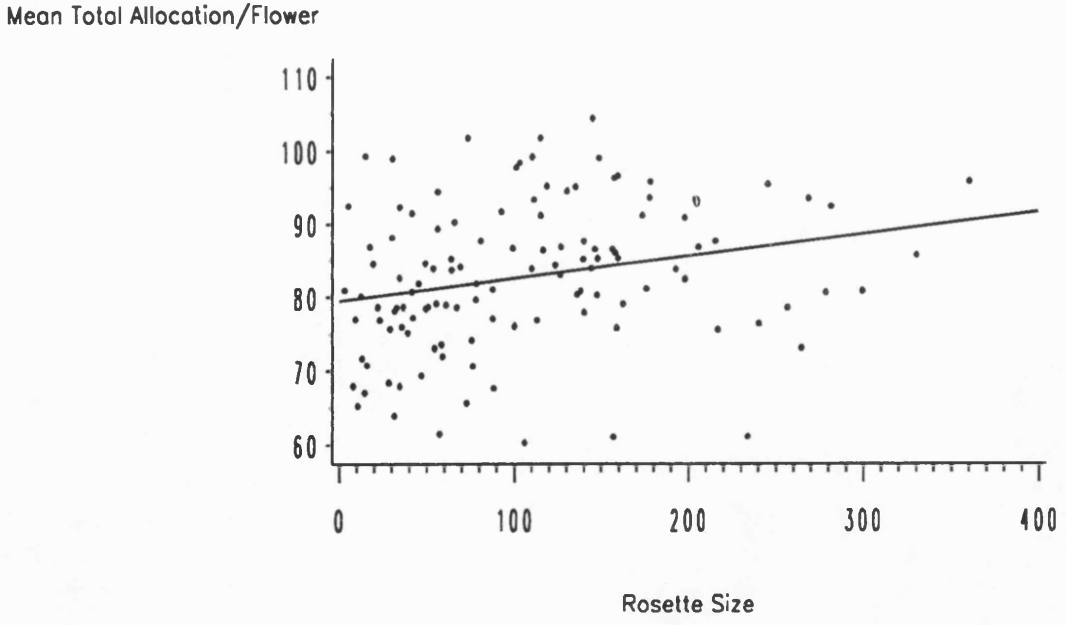


Fig 3-51 – Relationship Between Rosette Size Before Flowering And Mean Total Allocation To Reproduction/Flower



size. Such an effect is suggested by the results, although this is not significant. The regression coefficient for mean pollen production per plant is on the border of significance (i.e. just significant). These two factors might explain why a significant regression against rosette size was not found for mean P/O per plant. It cannot be concluded, therefore, that large plants are relatively more male, but only that they allocate more overall to gamete production.

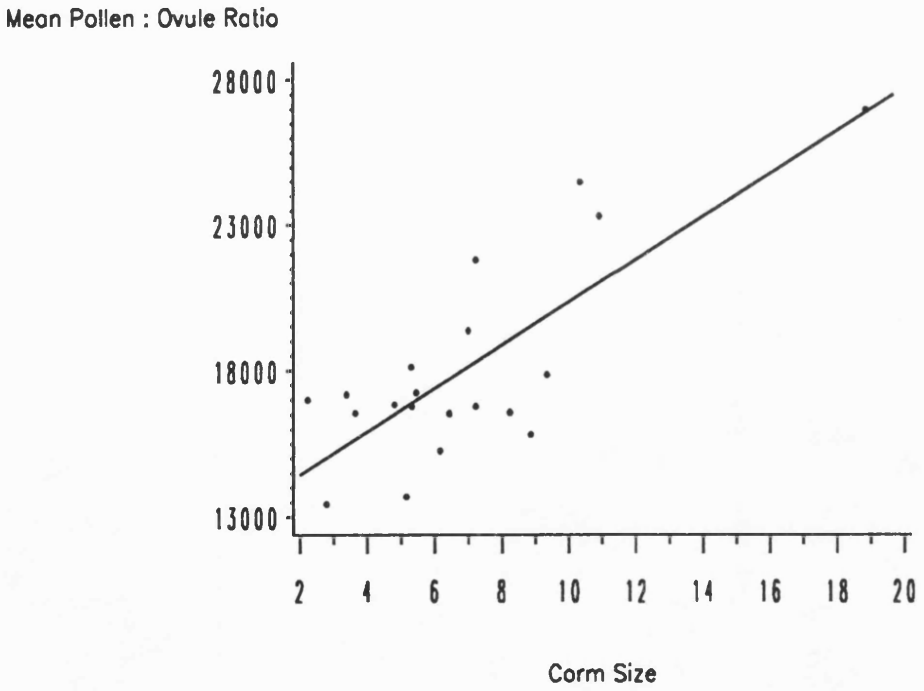
Regressing P/O against corm size after flowering revealed a significant positive regression. This indicates that a plant which allocates relatively more to male function than to female function is able to allocate more resources to its corm than a plant which allocates relatively more to female function than to male function. None of the other variables showed positive regressions with corm size. The results are summarized in table 3.17 and Fig. 3.52.

Regression analysis of the number of flowers produced by a plant during its flowering season (as a measure of total reproductive effort) against the mean G (Lloyd's measure of effective gender) per plant did not reveal a significant relationship ($p > 0.05$). When T_f was regressed against G, a highly significant positive relationship was found ($p < 0.01$). However, since the equation $T_f = g_i + a_iE$ is used in the calculation of both the x and y variables for this latter analysis, this is not

TABLE 3.17 REGRESSION ANALYSIS OF CORM SIZE AT THE END OF THE FLOWERING SEASON *VERSUS* ALL OTHER VARIABLES. FOR BLOCK 1 ONLY.

	REGRESSION ANALYSIS ON CORM SIZE *VERSUS*							
	MEAN OVULE COUNT	MEAN POLLEN COUNT	MEAN RATIO	MEAN TOTAL ALLOCATION PER FLOWER	TOTAL ALLOCATION PER PLANT	NUMBER OF FLOWERS PRODUCED	DURATION OF FLOWERING	ROSETTE SIZE
Regression coefficient	0.639	12955	738	0.232	161	1.85	0.149	3.19
Intercept value	43.1	593715	12962	81.1	9954	119	43.9	50.1
F	3.65	3.54	28.3	0.104	0.32	0.37	0.06	1.07
t	-1.91	1.88	5.32	0.32	-0.56	-0.61	0.24	1.03
Degrees of freedom	1,18	1,18	1,18	1,18	1,18	1.18	1.18	1,18
p	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Fig. 3.52– Relationship Between Corm Size After Flowering And Mean Pollen : Ovule Ratio Per Flower



surprising. It would be statistically unsound to use such an analysis alone to draw conclusions. If there had been some suggestion of a relationship between G and the number of flowers produced, the relationship between G and T_f may have backed this up. However, there was not. This, together with the results of the regression analyses on rosette size, indicates that there is no relationship between gender and total allocation to reproduction. These results are summarised in table 3.18 and Figs. 3.53 and 3.54.

iii) Discussion

The variables which were measured were chosen for particular reasons. It was originally hoped that more could be measured (e.g. pollen viability, seed set, height of the flowers and more flowers per plant). This proved to be impossible because of the unexpectedly high numbers of flowers produced by each plant and because of the number of plants involved. Because of statistical considerations, the number of plants investigated could only have been reduced by reducing the numbers of clones, not by reducing the number of replicates per clone. It was decided that, on balance, at this stage of the investigation, a more detailed investigation of a few clones would prove less informative than a less detailed investigation of a larger number of clones. Several of the measures used require further comment.

Corm size was measured to provide an estimate of the amount of resources a plant was able to store by the end of the flowering

TABLE 3.18 SUMMARY OF REGRESSION ANALYSIS PERFORMED TO FIND ANY RELATIONSHIP BETWEEN GENDER AND TOTAL ALLOCATION TO GAMETE PRODUCTION

	REGRESSION ANALYSIS PERFORMED ON	
	NUMBER OF FLOWERS *VERSUS* MEAN G PER PLANT	TOTAL ALLOCATION/ FLOWER *VERSUS* G
Regression coefficient	0.0001	0.0017
Intercept value	0.475	0.626
F	1.93	331.72
t	1.39	-18.24
Degrees of freedom	1,117	1,1068
p	>0.05	<0.001

Fig. 3·53– Relationship Between Number of Flowers Produced By A Plant And Mean G Per Plant

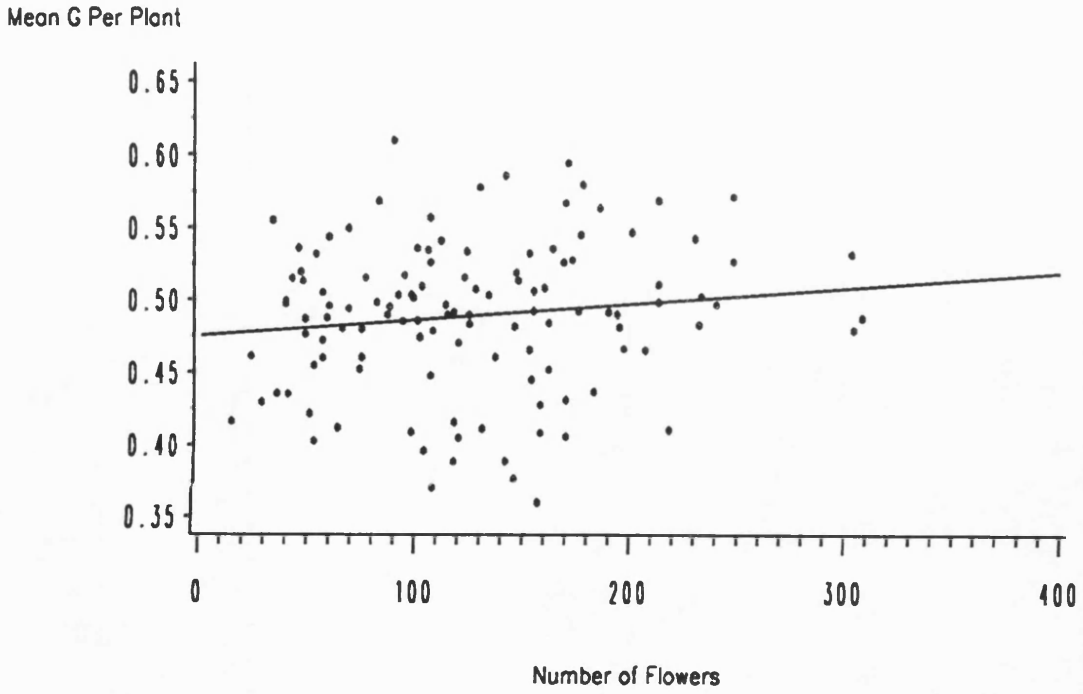
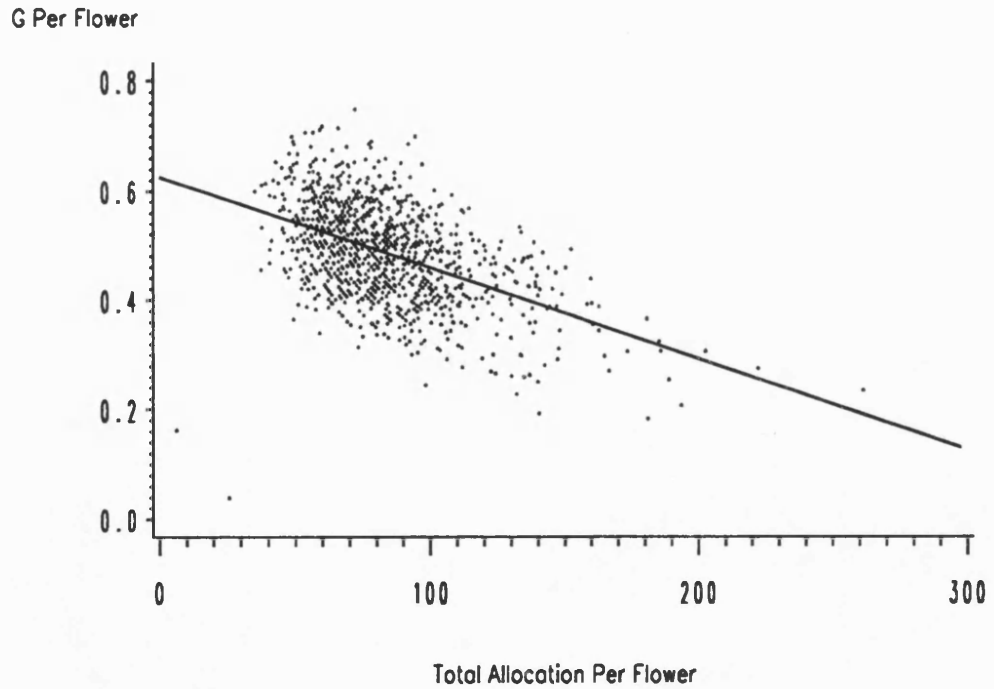


Fig. 3·54 – Relationship Between Total Allocation Per Flower And G Per Flower



season, and thus have available to initiate growth and reproduction in the following season.

The total number of flowers produced by a plant during its flowering season can be used as a rough estimate of total reproductive effort. This estimate was required to investigate the relationship between total reproductive effort and plant gender. It was also used to calculate T_p, which is a more accurate estimate of total reproductive effort per plant.

The duration of flowering does not necessarily reflect a plant's total reproductive effort, since a plant could, for example, produce 10 flowers per day for 6 days or one flower per day for 60 days. However, the duration of flowering has consequences for gene flow, which will be discussed in more detail in the next chapter.

Total pollen counts and total ovule counts per flower were used as an estimate of reproductive allocation to male and female function respectively. As has already been discussed in the introduction to this chapter, there are problems with this measure. For instance, its use assumes that all other reproductive structures are equally divided between male and female function. This may not be so (Bell, 1985). However, determining the extent which these other structures were contributed to by the male or female function was beyond the scope of this investigation. Consequently, it was decided to measure

only relative allocation to pollen and ovules. In measuring allocation to pollen and ovules, it would have been preferable to be able to measure viable pollen and ovules, as well as total pollen and ovules per flower. However, this proved impossible as many of the flowers had to be stored before they could be analysed. No storage method could be devised that did not have an effect on pollen or ovule viability.

Pollen/ovule ratio (P/O) gives an estimate of relative allocation to male and female function per flower or per plant. However, P/O does not take into account the relative allocation within the population as a whole. Lloyd's (1979, 1980) measure of G does this, providing an estimate of the portion of genes a plant is most likely to transmit through its male or female function. A G value of 0.5 indicates that a plant is equally likely to transmit genes through either sex. A value of >0.5 indicates that it will transmit more through its female function. A value of <0.5 indicates that it will transmit more through its male function.

Total allocation per flower and per plant gives a measure of the overall reproductive status of the plant relative to other plants. It is important to equate pollen and ovule counts before summing them to give total investment in gametes, as both the numbers and sizes of the two gamete types are very different. The equivalence factor (E) achieves this. It is based on the fact that an embryo is formed by one male gamete fertilizing one female

gamete. Therefore, the number of successful male gametes (which in this case are in abundance) is limited by, and equal to, the number of successful female gametes.

Mean values of pollen count per flower, ovule count per flower, P/O per flower, G per flower and total allocation per flower were used when performing the ANOVA, because allocation pattern varied through the season. The flowers from different plants were not necessarily picked on equivalent days. Thus, direct comparisons between first flower analysed, second flower analysed, etc., would have been meaningless. For instance, flowers 1, 7, 14, 21, 28, 35, 42, 49, 56 and 60 may have been used from a plant with a flowering season of 60 days. Whereas flowers from days 1, 5, 10, 15, 20, 25, 30, 35, 40 and 45 may have been used from a plant with a flowering season of 45 days. This would mean that the second flower analysed from each of these plants would not have been picked on either the same date or be the same flower number in the flowering sequence. Means were used to give a single more comparable value.

There are two possible ways of calculating the mean P/O per plant - calculating the mean of the ratios (equation 1) or the ratio of means (equation 2):

1) $$\frac{(\text{ratio 1} + \text{ratio 2} + \ldots \text{ratio 10})}{10}$$

2) $$\frac{(\text{total pollen count 1} + \text{total pollen count 2} + \ldots \text{total pollen count 10})}{(\text{total ovule count 1} + \text{total ovule count 2} + \ldots \text{total ovule count 10})}$$

The choice of which method to use depends upon the amount of intrinsic error there is in each P/O. When there is very little or no intrinsic error, the mean of ratios should be used, as any variation around the mean is due to actual variation in the P/O for different flowers, rather than variation in each P/O. It is the variation around the mean that we are interested in. When there is large intrinsic variation in P/O, the ratio of means should be used, as most of the variation around the mean can be accounted for by variation around each P/O rather than between P/Os. Here, both methods were used because it was not certain how much intrinsic variation there was in each P/O. It is the variation between P/Os that is interesting, so equation 1 was used to look at this. Equation 2 was used to back up the assumption that intrinsic variation was small. This it did, since there was relatively little variation between the two sets of values.

Competition was selected as a treatment because of the positioning and design of the experiment. The experiment was performed in cultivated ground at the research field station situated on the campus of the University of Bath. Under these conditions, it was not possible to experimentally control factors such as light, moisture, nutrient availability and temperature. It was therefore decided to stress the plants via competition. Conspecifics of the same clone were used to try to keep the competitive pressures as constant as possible between clones.

The absence of treatment effects for any of the variables indicates that *R. bulbosus* may not be able to alter its total or relative allocation to gender in response to environmental conditions. If it was able to, theory predicts that a switch towards maleness would have been induced by competition (see introduction to this chapter). However, it is by no means certain that the treatment had its desired effect of imposing competition! Neither the competed nor the control plants appear to have been placed under stress! Corm sizes measured for block 1 after flowering for both competed and control plants were large compared with those that have been seen at Rainbow Wood. Harper (1957) reports that large corms are found where the plants exist in good conditions. In addition, a large number of flowers was produced by all the plants (an overall mean of 124 flowers per plant). Clapham *et al* (1962) report a maximum of eight flowering stems in good conditions. The experimental plants produced many more flowers than this. These observations indicate that, rather than being under stress, all of the plants were growing under non-stressful conditions. This, in turn, suggests that the treatment applied was not harsh enough to induce stress. Consequently, the conclusions to be reached from the competition treatment should be treated with great caution.

The significant differences between clones in both total and relative allocation to gametes indicates a genetic component to reproductive allocation. There were also significant differences in plant size before flowering between clones. This could also be

due to genetic differences between plants. It could, however, be a result of effects caused by the different environments from which the plants were originally collected. Plants from areas with, for example, poor soil may, as a result, be much smaller in the following years. Plants were kept in very similar conditions during the year preceding the setting up of the experiment in an attempt to neutralise such effects. They were also planted out well before any measurements were made. However, there may still have been some such effects. Running the experiment for several years would have shown if this was the case. However, this was not possible. Independent of whether the effect is genetic or environmental, the clonal difference in plant size before flowering may be of some importance in resource dependent allocation strategies. For instance, a relatively small clone type may have fewer resources overall to allocate to gamete production. This theory is supported by the positive linear relationship between total allocation to gamete production and rosette size before flowering. It may then be predicted from theory (Charnov, 1982) and the conclusions of previous studies (Lovett-Doust and Cavers, 1982 and Policansky, 1981) that such a plant will produce relatively more male gametes than female gametes (i.e. have a relatively high P/O and relatively low G value). This pattern does seem to be followed in clone 9, whose total allocation to gamete production is lower than that for all other clones except clone 8 (this difference was not found to be significant using the MSD method). Clone 9 also has a significantly higher mean P/O than clones 2 and 10. However,

clone 8, which has significantly lower allocation to gametes than clone 6, and a lower (although not significantly so) allocation than all other clones, has a significantly lower mean P/O than clones 1, 2, 3, 5 and 6. This contradicts these predictions. Both have G values of <0.5. Clone 6, however, also fulfills the predictions in having the highest overall allocation to gametes and a relatively low P/O (significantly lower than clones 3 and 7). It also has a G value >0.5. See tables 3.7 - 3.10 for a summary of these results. There is no clear pattern across clones. This, together with the lack of a significant relationship between plant size before flowering and relative allocation to pollen and ovules or between total reproductive effort and G, indicates that the predictions made are not generally fulfilled, although this may be the strategy adopted by some plants.

The results are slightly confusing and it is difficult to find consistent patterns emerging. The only clear conclusions which can be drawn are that different clones adopt different patterns in reproductive allocation. Also it would appear that a larger plant (before flowering) will allocate more overall to gamete production. A plant which is more male during the flowering season will tend to have a larger corm size at the end of the flowering season. In addition, overall, 7 clones became relatively more female with time, whilst 3 remained constant in terms of their gender allocation strategy. All clones allocated less to reproduction with time.

Many other, possibly significant, factors may have been operating on the plants during the experiment. This may have masked some of the underlying patterns. For instance, the experimental plants were not all cloned on the same day, due to the large numbers of plants required. Soil quality, light and drainage may have varied within the experimental plot, even though the experiment was designed to minimise such effects. The sample size of 10 clones was relatively small (a compromise between the ideal and the feasible). Including more clones may have revealed more obvious patterns.

It was noted, when carrying out pollen and ovule counts, that there was some variation in these through time. To increase sample size when studying this effect, it was decided to group data across blocks and treatments. This is a reasonable approach, as no significant block or treatment effects were found from the ANOVA. Regression analyses were performed against time elapsed since the production of the first flower by each plant as this, rather than time elapsed from a particular date, appeared to be the important factor causing variation.

The significant regressions obtained demonstrate that the number of gametes produced by a plant declines as its flowering season progresses. Significant differences between clones were revealed by this technique. This lends further support to the conclusions drawn from the ANOVA that there is a significant genetic effect in allocation strategy. In this case, the clones

are adopting different strategies in the way they allocate resources to reproduction through the flowering season. For instance, clone 9 decreases its relative allocation to pollen production (as indicated by P/O) at a significantly faster rate than any of the other clones. That is, it is becoming increasingly more female significantly faster than any of the other clones. Clones 1, 4 and 6, which did not significantly change their relative allocation to pollen and ovules, unlike all the other clones, are adopting a fairly constant strategy through the season, although decreasing their total allocation. The fact that there was no significant difference between the regression coefficients for data pooled by treatment and plotted against time, strengthens the evidence for concluding that the treatment applied did not affect allocation strategy, either overall or in the way resources are allocated through the flowering season.

Because the regression analyses on time were found to be significant, it was decided to perform an ANCOVA on the data. In removing some of the inherent variation in the data caused by time, it was hoped that any small treatment effects might become apparent. This was not the case.

d) An experiment to determine the effect of water-logging and lack of nutrients on the sexual allocation strategy of *R. bulbosus*

i) Methods

This experiment was conducted on a much smaller scale to the one previously described, in order to allow more measurements to be made on each plant. The design used was a single randomized block.

Five clones were used and three treatments, with three replicates per clone per treatment. The five clone types used were clones 1, 2, 3, 6 and 10 from the previous experiment. These are given the same numbers here.

The plants used were propagated at the end of March 1988. Since all plants were initiated from cuttings of equal sizes, at the same time, they started off on a more equal basis than those plants in the previous year's experiment. This reduced variation inherent in the experiment. Once cuttings had produced roots and shoots, they were potted into their treatment pots. The treatments were:

i) deprivation of nutrients by growing plants in sand;

ii) water-logging;

iii) control plants grown in 3.5" pots of John Innes M2 compost which was kept damp.

The experiment commenced at the beginning of May 1988. The plants were laid out in a plot 7 m x 7 m, at a spacing of 0.5 m between

plants. A guard row of plants was placed around the experimental plot. Due to vandalism, the experiment had to be moved to a different location soon after its initiation, and two plants had to be replaced. The final location of the experimental plot was an area between two of the university greenhouses.

The plot was visited daily during the flowering season, and the following measurements were taken:

i) the height of each flower on the day before opening was measured to the nearest half centimetre;

ii) every other flower produced by each plant (starting with the first flower produced by each plant) was collected. Counts of total pollen, viable pollen and the number of ovules were obtained from these;

iii) the remaining flowers were labelled with the date of opening and left to set seed. Once seed had set (about six weeks after opening) percentage seed set per flower was calculated;

iv) total number of flowers produced by each plant;

v) duration of flowering for each plant;

vi) corm size after flowering.

Several calculations were performed on the data:

i) mean proportion of seeds set per plant;

ii) 'effective' ovule production (vO) was calculated by multiplying the mean proportion of seeds set per plant by the number of ovules in each of its flowers;

iii) total pollen/total ovule ratio (P/O);

iv) viable pollen/'effective' ovule ratio (vP/O);

v) the sum of the total pollen counts was divided by the sum of the ovule counts for each flower (M);

vi) the sum of the viable pollen counts was divided by the sum of the 'effective' ovule counts for each flower (vM);

vii) total allocation to gametes per flower (T_f) was calculated using the methods described in the previous section;

viii) total allocation to viable gametes per flower (vT_f) was calculated using the methods described in the previous section, but substituting viable pollen and 'effective' ovules for total pollen and ovules;

ix) total allocation to gametes per plant (T_p) was calculated by multiplying mean T_f per plant by the total number of flowers produced by that plant;

x) total allocation to viable gametes per plant (vT_p) was calculated as for T_p but using vT_f instead of T_f;

xi) G (Lloyd 1979, 1980) per flower was calculated using the methods described in the previous section;

xii) viable G per flower was calculated using the methods described in the previous section, but substituting viable pollen and 'effective' ovules for total pollen and ovules;

xiii) means of ovule number per flower, total pollen count per flower, P/O per flower, vP/O per flower, G per flower and viable G per flower were calculated for each plant.

Several statistical tests were carried out using Genstat (version 5) and Minitab. Genstat was used to perform a two-way ANOVA on $\log_{10}$'s of mean total pollen count per flower, mean viable pollen count per flower, mean ovule number per flower, mean 'effective' ovule number per flower, mean P/O per flower, mean vP/O per flower, M, vM, T_p, vT_p, mean G per flower, mean viable G per flower, number of flowers produced per plant, duration of flowering per plant, corm size after flowering and mean seed set per flower. Log_{10} of the values was used to normalise the data.

Minitab was used to perform regression analyses on height of flower *versus* the following: seed set per flower, total ovule count per flower, 'effective' ovule count per flower, total pollen count per flower, viable pollen count per flower, P/O per flower, vP/O per flower, T_f, vT_f, G per flower and viable G per flower. Minitab was also used to carry out regression analyses on corm size after flowering against all other variables for which an ANOVA was performed.

Mean G per flower per plant and mean viable G per flower per plant were regressed on number of flowers produced per plant.

ii) Results

The ANOVA revealed no significant differences between the five clones in their total allocation to gametes, flower production, or corm size after flowering. However, a significant clone effect was found for mean G per flower when the viability of

gametes is taken into account. This effect was not revealed when total gametes are used to calculate G. If the table of means for clones (table 3.21) is studied, it shows that the value of G for clone 6 falls from 0.182 to 0.074 when account is taken of gamete viability. This clone has a significantly lower G value than clones 1 and 3 when the minimum significant difference (Sokal and Rohlf, 1981) is calculated. It appears, therefore, that the significant clone effect for mean viable G is due mainly to clone 6. Clone 6 has the lowest seed set value of all five clones, although this is not reflected in a significant clone effect for seed set in the ANOVA.

A significant treatment effect was revealed for mean number of ovules per flower, the ratio of means for total (rather than viable) gametes, total gametes per plant, total viable gametes per plant, number of flowers produced per plant, duration of a plant's flowering season and mean G per flower when this is calculated using total, rather than viable, gametes. The treatment effect was analysed further by comparing the treatments within the structure of the ANOVA. Studying the tables of means by treatment (table 3.20) it is apparent that the mean values for treatments 2 and 3, for all variables, are very similar. To discover whether there is any difference between these two treatments, the treatment sum of squares can be further partitioned into the sum of squares for treatments 2 and 3 and the sum of squares for treatment 1. The variance ratios (F) can then be calculated for treatments 2 and 3 and for treatment 1. The F values prove to be

significant ($p < 0.05$) for treatment 1, but not significant ($p > 0.05$) for treatments 2 and 3. This shows that treatments 2 and 3 are not significantly different to each other. Where there is an overall treatment effect, therefore, this is due to treatment 1, which must be significantly different to the other two treatments.

The clone by treatment effects are not consistent. It would appear, therefore, that clones are reacting in different ways to the treatments applied. For instance, clone 2 produced no flowers when either treatment 1 or 2 was applied. Clone 1, on the other hand, produced more flowers when subjected to treatments 1 or 2 than when subjected to treatment 3. Treatment 1 resulted in clones 2, 3 and 10 producing no flowers at all. This was also the case with treatment 2 for clone 2 and with treatment 3 for clone 6. The results of the ANOVA are summarised in tables 3.19 - 3.22.

When regressed against corm size after flowering, none of the measured variables showed a significant relationship. These results are summarised in table 3.23. However, a significant ($p < 0.01$) negative regression was demonstrated when the height of the flower above ground level was compared with the total pollen count per flower, and a significant ($p < 0.01$) positive regression found for G regressed on flower height. None of the other measured variables show a significant relationship when regressed

against height of flower. These results are summarised in table 3.24.

There were too few flowers produced per plant to carry out regression analyses using time as one of the variables. This also meant that no comparisons could be made between plants in the way in which they allocate resources through the season.

The regressions of mean G per flower per plant and mean viable G per flower per plant against number of flowers produced by a plant did not give significant results ($F = 1.11$, $p > 0.05$ and $F = 0.02$, $p > 0.05$ respectively).

iii) Discussion

The lack of a significant clone effect for most variables appears to contradict the results of the previous year's experiment. There was some evidence of a clone effect on the variable G, accounted for mainly by clone 6. In this experiment, only five of the original ten clones used in the previous experiment were also used in this experiment. The five clones were chosen at random, and at the time it was not known how much variation there was between them, as the data from the previous experiment had not been fully analysed before this experiment was initiated (due to the length of time taken to determine pollen counts). Now that the results of the previous experiment are available, it can be seen that significant differences between the five clones were revealed for certain variables (mean ovule

TABLE 3.19/I RESULTS OF THE ANALYSIS OF VARIANCE FOR ALL VARIABLES MEASURED

	CLONE EFFECTS		TREATMENT EFFECTS		CLONE X TREATMENT EFFECTS	
	F	$P_{(4,28)}$	F	$P_{(2,28)}$	F	$P_{(8,28)}$
MEAN NUMBER OF OVULES PER FLOWER	0.90	>0.05	3.81	<0.05	4.17	<0.01
MEAN NUMBER OF 'EFFECTIVE' OVULES PER FLOWER	2.33	>0.05	2.79	>0.05	4.48	<0.01
MEAN POLLEN COUNT PER FLOWER	1.20	>0.05	3.12	>0.05	3.88	<0.01
MEAN VIABLE POLLEN COUNT PER FLOWER	1.12	>0.05	3.19	>0.05	3.88	<0.01
MEAN RATIO PER FLOWER	1.27	>0.05	2.92	>0.05	3.73	<0.01
MEAN VIABLE RATIO PER FLOWER	0.95	>0.05	3.28	>0.05	3.52	<0.01
TOTAL POLLEN PER PLANT / TOTAL OVULES PER PLANT	1.01	>0.05	3.51	<0.05	3.99	<0.01
TOTAL VIABLE POLL. PER PLANT / TOTAL 'EFFEC.' OV. PER PLANT	1.20	>0.05	3.25	>0.05	3.71	<0.01

NOTE: ANOVA was performed using $\log_{10}$ of all values to normalise the data.

TABLE 3.19/II RESULTS OF THE ANALYSIS OF VARIANCE FOR ALL VARIABLES MEASURED

	CLONE EFFECTS		TREATMENT EFFECTS		CLONE X TREATMENT EFFECTS	
	F	$P_{(4,28)}$	F	$P_{(2,28)}$	F	$P_{(8,28)}$
TOTAL GAMETES PER PLANT	0.78	>0.05	4.66	<0.05	3.65	<0.01
TOTAL VIABLE GAMETES PER PLANT	1.97	>0.05	5.89	<0.01	1.98	>0.05
NUMBER OF FLOWERS PER PLANT	1.03	>0.05	5.39	<0.01	2.87	<0.05
DURATION OF FLOWERING PER PLANT	0.26	>0.05	4.43	<0.01	3.25	<0.01
CORM SIZE AFTER FLOWERING PER PLANT	2.10	>0.05	1.10	>0.05	0.58	>0.05
MEAN SEED SET PER FLOWER	2.28	>0.05	2.05	>0.05	3.68	<0.01
MEAN G PER FLOWER	0.90	>0.05	3.74	<0.05	4.71	<0.01
MEAN VIABLE G PER FLOWER	3.25	<0.05	2.34	>0.05	6.00	<0.01

TABLE 3.20/I ANOVA TABLE OF MEANS BY TREATMENT

	TREATMENT 1	TREATMENT 2	TREATMENT 3
MEAN NUMBER OF OVULES/FLOWER	5.8[b]	19.0[a]	17.9[a]
MEAN NUMBER OF 'EFFECTIVE' OVULES PER FLOWER	2.63	5.27	8.13
MEAN POLLEN COUNT/FLOWER	179033	469027	482018
MEAN VIABLE POLLEN COUNT PER FLOWER	99781	339578	347443
MEAN RATIO PER FLOWER	8211	15248	35974
MEAN VIABLE RATIO PER FLOWER	11318	87379	30935
TOTAL POLLEN PER PLANT / TOTAL OVULES PER PLANT	12.5[b]	36.3[a]	37.0[a]
TOTAL VIABLE POLL. PER PLANT / TOTAL 'EFFEC.' OV. PER PLANT	4.5	11.7	14.7

Note: Superscripts indicate significant differences between means.

TABLE 3.20/II ANOVA TABLE OF MEANS BY TREATMENT

	TREATMENT 1	TREATMENT 2	TREATMENT 3
TOTAL GAMETES PER PLANT	24[b]	214[a]	230[a]
TOTAL VIABLE GAMETES PER PLANT	0.40[b]	1.19[a]	1.13[a]
NUMBER OF FLOWERS PRODUCED PER PLANT	0.53[b]	3.53[a]	3.67[a]
DURATION OF FLOWERING PER PLANT	1.87[b]	7.40[a]	7.60[a]
CORM SIZE AFTER FLOWERING PER PLANT	0.454	0.525	0.600
MEAN SEED SET PER FLOWER	12.1[b]	17.1[a]	23.7[a]
MEAN G PER FLOWER	0.127[b]	0.315[a]	0.260[a]
MEAN VIABLE G PER FLOWER	0.157	0.262	0.298

Note: Superscripts indicate significant differences between means.

TABLE 3.21/I ANOVA TABLE OF MEANS BY CLONE

	CLONE 2	CLONE 1	CLONE 3	CLONE 6	CLONE 10
MEAN NUMBER OF OVULES/FLOWER	14.2	15.8	17.6	11.1	12.6
MEAN NUMBER OF 'EFFECTIVE' OVULES PER FLOWER	5.20	6.89	9.65	1.20	3.77
MEAN POLLEN COUNT/FLOWER	280890	492498	470497	251227	388350
MEAN VIABLE POLLEN COUNT PER FLOWER	206269	324319	295777	213594	271378
MEAN RATIO PER FLOWER	7855	20757	27484	7920	35038
MEAN VIABLE RATIO PER FLOWER	14118	35402	21578	110834	34121
TOTAL POLLEN PER PLANT / TOTAL OVULES PER PLANT	26.6	34.0	35.0	20.4	27.0
TOTAL VIABLE POLL. PER PLANT / TOTAL 'EFFEC.' OV. PER PLANT	9.1	13.0	15.3	5.3	8.9

Note: Superscripts indicate significant differences between means.

TABLE 3.21/II ANOVA TABLE OF MEANS BY CLONE

	CLONE 2	CLONE 1	CLONE 3	CLONE 6	CLONE 10
TOTAL GAMETES PER PLANT	124	74	201	102	280
TOTAL VIABLE GAMETES PER PLANT	0.68	1.04	1.18	0.61	1.01
NUMBER OF FLOWERS PRODUCED PER PLANT	1.67	1.44	3.33	1.67	4.78
DURATION OF FLOWERING PER PLANT	5.00	4.00	6.11	6.00	7.00
CORM SIZE AFTER FLOWERING PER PLANT	0.582	0.688	0.435	0.434	0.494
MEAN SEED SET PER FLOWER	12.2	29.4	29.9	4.1	12.4
MEAN G PER FLOWER	0.197	0.314	0.276	0.182	0.200
MEAN VIABLE G PER FLOWER	0.200^{ab}	0.357^{b}	0.352^{b}	0.074^{a}	0.213^{ab}

Note: Superscripts indicate significant differences between means.

TABLE 3.22/I ANOVA TABLE OF MEANS BY CLONE BY TREATMENT

	CLONE 2			CLONE 1			CLONE 3		
	TRT. 1	TRT. 2	TRT. 3	TRT. 1	TRT. 2	TRT. 3	TRT. 1	TRT. 2	TRT. 3
MEAN NUMBER OF OVULES/FLOWER	0	0	42.6	20.7	17.5	9.2	0	32.2	20.5
MEAN NUMBER OF 'EFFECTIVE' OVULES PER FLOWER	0	0	15.61	10.75	3.68	6.23	0	13.34	15.61
MEAN POLLEN COUNT/FLOWER	0	0	842669	661840	458118	357535	0	761110	650381
MEAN VIABLE POLLEN COUNT PER FLOWER	0	0	618807	300114	334045	338798	0	438015	449316
MEAN RATIO PER FLOWER	0	0	23565	31863	17337	13071	0	24329	58123
MEAN VIABLE RATIO PER FLOWER	0	0	42354	28635	59330	18241	0	33902	30832
TOTAL POLLEN PER PLANT / TOTAL OVULES PER PLANT	0	0	79.9	45.2	34.5	22.4	0	60.4	44.6
TOTAL VIABLE POLL. PER PLANT / TOTAL 'EFFEC.' OV. PER PLANT	0	0	27.4	16.4	10.0	12.7	0	21.7	24.1

TABLE 3.22/II ANOVA TABLE OF MEANS BY CLONE BY TREATMENT

	CLONE 2			CLONE 1			CLONE 3		
	TRT. 1	TRT. 2	TRT. 2	TRT. 1	TRT. 2	TRT. 3	TRT. 1	TRT. 2	TRT. 3
TOTAL GAMETES PER PLANT	0	0	372	70	85	67	0	343	260
TOTAL VIABLE GAMETES PER PLANT	0	0	15.0	6.0	2.0	4.0	0	11.0	7.33
NUMBER OF FLOWERS PRODUCED PER PLANT	0	0	5.0	1.67	1.67	1.0	0	5.67	4.33
DURATION OF FLOWERING PER PLANT	0	0	2.03	1.39	1.05	0.69	0	2.09	1.47
CORM SIZE AFTER FLOWERING PER PLANT	0.427	0.618	0.701	0.576	0.616	0.871	0.391	0.547	0.367
MEAN SEED SET PER FLOWER	0	0	36.7	51.1	14.3	22.7	0	41.7	48.0
MEAN G PER FLOWER	0	0	0.590	0.466	0.341	0.136	0	0.539	0.290
MEAN VIABLE G PER FLOWER	0	0	0.600	0.655	0.253	0.164	0	0.636	0.419

TABLE 3.22/III ANOVA TABLE OF MEANS BY CLONE BY TREATMENT

	CLONE 6			CLONE 10		
	TRT. 1	TRT. 2	TRT. 3	TRT. 1	TRT. 2	TRT. 3
MEAN NUMBER OF OVULES/FLOWER	8.5	24.8	0	0	20.3	17.5
MEAN NUMBER OF 'EFFECTIVE' OVULES PER FLOWER	2.38	1.22	0	0	8.11	3.19
MEAN POLLEN COUNT/FLOWER	233323	520359	0	0	605547	559504
MEAN VIABLE POLLEN COUNT PER FLOWER	198789	441993	0	0	483839	330294
MEAN RATIO PER FLOWER	9193	14569	0	0	20006	85110
MEAN VIABLE RATIO PER FLOWER	27957	304546	0	0	39117	63247
TOTAL POLLEN PER PLANT / TOTAL OVULES PER PLANT	17.1	44.1	0	0	42.7	38.2
TOTAL VIABLE POLL. PER PLANT / TOTAL 'EFFEC.' OV. PER PLANT	6.2	9.6	0	0	17.3	9.5

TABLE 3.22/IV ANOVA TABLE OF MEANS BY CLONE BY TREATMENT

	CLONE 6			CLONE 10		
	TRT. 1	TRT. 2	TRT. 3	TRT. 1	TRT. 2	TRT. 3
TOTAL GAMETES PER PLANT	51	254	0	0	386	452
TOTAL VIABLE GAMETES PER PLANT	3.33	14.67	0	0	9.33	11.67
NUMBER OF FLOWERS PRODUCED PER PLANT	1.0	4.0	0	0	6.33	8.0
DURATION OF FLOWERING PER PLANT	0.58	1.25	0	0	1.57	1.46
CORM SIZE AFTER FLOWERING PER PLANT	0.354	0.464	0.485	0.521	0.381	0.579
MEAN SEED SET PER FLOWER	9.3	3.0	0	0	26.3	11.0
MEAN G PER FLOWER	0.167	0.378	0	0	0.318	0.283
MEAN VIABLE G PER FLOWER	0.131	0.090	0	0	0.331	0.309

TABLE 3.23/I RESULTS OF REGRESSIONS OF ALL VARIABLES AGAINST CORM SIZE AFTER FLOWERING

	CORM SIZE REGRESSED AGAINST						
	MEAN OVULE PER FLOWER	MEAN 'EFFEC-TIVE' OVULE PER FLOWER	MEAN POLLEN PER FLOWER	MEAN VIABLE POLLEN PER FLOWER	MEAN RATIO PER FLOWER	MEAN VIABLE RATIO PER FLOWER	TOTAL POLLEN/ TOTAL OVULE
REGRESSION COEFFICIENT	-3.6	-2.95	-264339	-207360	-31826	-26674	-12.6
INTERCEPT ON 'Y' AXIS	16.1	6.89	515886	371457	36569	57256	35.2
F	0.114	0.365	0.944	0.991	1.567	0.151	0.368
t	-0.34	-0.60	-0.97	-1.00	-1.25	-0.39	-0.61
$P_{(1,44)}$	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

TABLE 3.23/II RESULTS OF REGRESSIONS OF ALL VARIABLES AGAINST CORM SIZE AFTER FLOWERING

	CORM SIZE REGRESSED AGAINST						
	TOTAL VIABLE POLL./ TOTAL 'EFFEC-TIVE' OVULE	TOTAL GAMETES PER PLANT	TOTAL VIABLE GAMETES PER PLANT	NUMBER OF FLOWERS PRODUCED PER PLANT	DURATION OF FLOWERING PER PLANT	MEAN G PER FLOWER	MEAN VIABLE G PER FLOWER
REGRESSION COEFFICIENT	-6.89	-144	-54.4	-2.65	-5.89	-0.162	+0.087
INTERCEPT ON 'Y' AXIS	14.0	232	81.2	3.97	8.72	0.420	0.469
F	0.69	0.942	1.224	1.128	1.457	3.64	0.193
t	-0.83	-0.97	-1.11	-1.06	-1.21	1.91	0.44
$P_{(1,44)}$	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

TABLE 3.24/I RESULTS OF REGRESIONS OF ALL VARIABLES AGAINST HEIGHT OF FLOWER

	HEIGHT OF FLOWER REGRESSED AGAINST				
	SEED SET PER FLOWER	NUMBER OF OVULES PER FLOWER	NUMBER OF 'EFFECTIVE' OVULES PER FLOWER	POLLEN COUNT PER FLOWER	VIABLE POLLEN COUNT PER FLOWER
REGRESSION COEFFICIENT	0.010	0.383	0.175	-17213	-11952
F	1.85	2.34	1.295	9.482	2.505
t	1.36	1.53	1.14	-3.08	-1.58
P*	>0.05	>0.05	>0.05	<0.01	>0.05

* Degrees of freedom are 1,66 for all variables except seed set, where degrees of freedom are 1,43.

TABLE 3.24/II RESULTS OF REGRESSIONS OF ALL VARIABLES AGAINST HEIGHT OF FLOWER

	HEIGHT OF FLOWERS REGRESSED AGAINST					
	RATIO PER FLOWER	VIABLE RATIO PER FLOWER	TOTAL GAMETES PER FLOWER	TOTAL VIABLE GAMETES PER FLOWER	G PER FLOWER	VIABLE G PER FLOWER
REGRESSION COEFFICIENT	-4936	-2054	-0.253	-0.052	0.01	0.007
F	2.029	0.399	0.409	0.048	12.00	1.40
t	-1.42	-0.63	-0.64	-0.22	3.46	1.18
P*	>0.05	>0.05	>0.05	>0.05	<0.01	>0.05

* Degrees of freedom are 1,66 for all variables except seed set, where degrees of freedom are 1,43.

number per flower, mean P/O per flower and mean G per flower). Clone 3, in particular, was different from the other clones. It had a higher mean ovule number per flower, a higher mean ratio per flower, and a lower mean G per flower. So, significant clone effects might well be expected for these variables in this experiment as well. This was the case for mean G per flower (although this effect was mainly due to clone 6, not clone 3) but not for the other two variables. The clones used in this experiment had been kept under 'standard' conditions for a further year. Thus, any differences between clones resulting from original environmental effects may have been further reduced, resulting in less variation between clones from different environments. Plants may also have been reacting differently to the two very different environments in which the two experiments were performed. In the previous experiment, conditions were likely to be generally more variable than in this experiment, where conditions within treatments were kept much more constant. Unlike the previous experiment, where larger numbers of plants were required, it was possible, for this experiment, to grow plants from cuttings of the same size, taken at the same time and grown on in standard conditions for the same length of time. This may also have contributed to the contradiction.

Some differences between clones are revealed by the significant clone by treatment effects. These reveal that the clones are behaving in different ways in response to the treatments.

The clone by treatment effects need to be considered when drawing conclusions about overall treatment and clone effects. If clones respond in opposite ways to the treatments, there will be no overall treatment effect, since there will be a balance between the two responses. The same principle applies with clone effects. So, for each clone, the treatment has an effect but the nature of this effect is different for the five clones. This may be a contributing factor to the lack of an overall significant treatment effect for some variables.

Even taking these facts into account, it can be concluded that growing plants in sand reduced overall gamete production. It was thought that sand would contain fewer available nutrients, thus stressing the plants and reducing the resources available for reproduction. This does seem to be the case. It might also be expected, from theoretical considerations (see introduction to this chapter) that relative investment in male and female gametes would be affected by stress. Reduced soil quality has been implicated in increased relative investment in male function in a number of studies. Some of these are summarised in Charnov (1982), Freeman *et al* (1980) and Heslop-Harrison (1957). When including all data in the analyses, the experiment appears to show the opposite. That is, the P/O data indicate an increase in relative investment in the female function under stressful conditions!

However, if the value of G is considered, it would appear that the predictions have been fulfilled. Mean G is significantly lower for treatment 1 than for the other two treatments. That is, the plants are investing relatively more in the male function when under stress.

These two sets of results appear contradictory. However, this problem can be resolved by studying the tables of means (tables 3.20 - 3.22). There are several values of zero for all variables (except corm size) when no flowers were produced by a plant. In the first analysis, all of these values were included. For treatment 1, only four out of a total of fifteen plants flowered. For treatments 2 and 3, nine out of fifteen and eight out of fifteen plants flowered respectively. So, all mean values for treatment 1 would be reduced to a greater extent than those for treatments 2 and 3 simply because fewer plants actually flowered under treatment 1. It is important to include plants which did not flower in any analyses of total investment. When a plant did not flower, the total investment in sexual reproduction was zero. However, when looking at relative investment, these values of zero are meaningless, since a plant cannot relatively invest in gametes which it is not producing. So, the ANOVA was repeated for all measures of relative investment in male and female gametes, replacing the zeros with missing values. The results are contained in tables 3.25 - 3.28.

TABLE 3.25 RESULTS OF THE ANALYSIS OF VARIANCE ON MEASURES OF RELATIVE INVESTMENT, EXCLUDING THOSE PLANTS WHICH DID NOT FLOWER

	CLONE EFFECTS		TREATMENT EFFECTS		CLONE X TREATMENT EFFECTS	
	F	$P_{(4,19)}$	F	$P_{(2,19)}$	F	$P_{(5,19)}$
MEAN RATIO PER FLOWER	3.48	<0.05	4.94	<0.05	0.25	>0.05
MEAN VIABLE RATIO PER FLOWER	42.69	<0.001	59.18	<0.001	1.63	>0.05
TOTAL POLLEN/PLANT / TOTAL OVULE/PLANT	4.63	<0.01	22.68	<0.001	1.46	>0.05
TOTAL VI. POLL./PLANT / TOTAL 'EFF.' OV./PLANT	2.42	>0.05	4.37	<0.05	7.01	<0.01
MEAN G PER FLOWER	7.36	<0.01	5.15	<0.05	0.12	>0.05
MEAN VIABLE G PER FLOWER	62.25	<0.001	81.54	<0.001	3.82	<0.05

TABLE 3.26 ANOVA TABLE OF MEANS BY TREATMENT EXCLUDING THOSE PLANTS WHICH DID NOT FLOWER

	TREATMENT 1	TREATMENT 2	TREATMENT 3
MEAN RATIO PER FLOWER	29085[c]	14845[b]	69132[a]
MEAN VIABLE RATIO PER FLOWER	69111[b]	144892[a]	111226[a]
TOTAL POLLEN/PLANT / TOTAL OVULE/PLANT	48.9[b]	64.1[a]	68.0[a]
TOTAL VI. POLL./PLANT / TOTAL 'EFF.' OV./PLANT	16.4[b]	19.4[a]	28.2[a]
MEAN G PER FLOWER	0.495[a]	0.553[b]	0.466[a]
MEAN VIABLE G PER FLOWER	0.738[b]	0.450[a]	0.466[a]

NOTE: Superscripts indicate significant differences between means.

TABLE 3.27 ANOVA TABLE OF MEANS BY CLONE, EXCLUDING THOSE PLANTS WHICH DID NOT FLOWER

	CLONE 2	CLONE 1	CLONE 3	CLONE 6	CLONE 10
MEAN RATIO PER FLOWER	7323	33998	51201	36639	73922
MEAN VIABLE RATIO PER FLOWER	3037	69438	28811	270032	4054
TOTAL POLLEN/PLANT / TOTAL OVULE/PLANT	72.3	55.1	58.0	61.0	55.3
TOTAL VI. POLL./PLANT / TOTAL 'EFF.' OV./PLANT	20.5	23.6	26.5	18.6	17.6
MEAN G PER FLOWER	0.627	0.466	0.480	0.510	0.442
MEAN VIABLE G PER FLOWER	0.678	0.475	0.740	0.276	0.605

TABLE 3.28/I ANOVA TABLE OF MEANS BY CLONE BY TREATMENT, EXCLUDING PLANTS WHICH DID NOT FLOWER

	CLONE 2			CLONE 1			CLONE 3		
	TRT. 1	TRT. 2	TRT. 3	TRT. 1	TRT. 2	TRT. 3	TRT. 1	TRT. 2	TRT. 3
MEAN P/O PER FLOWER	15612	29922	23565	31863	29378	40752	42801	24329	86473
MEAN VIABLE P/O PER FLOWER	132315	80850	42354	28635	109564	70117	158908	33902	38573
TOTAL POLLEN/PLANT / TOTAL OVULE/PLANT	60.9	76.1	79.9	45.2	53.4	66.6	46.6	60.4	67.1
TOTAL VI. POLL./PLANT / TOTAL 'EFF.' OV./PLANT	15.6	18.5	27.4	16.4	16.4	38.0	21.6	21.7	36.2
MEAN G PER FLOWER	0.616	0.674	0.590	0.466	0.516	0.417	0.470	0.539	0.432
MEAN VIABLE G PER FLOWER	0.861	0.573	0.600	0.655	0.344	0.426	0.924	0.636	0.622

TABLE 3.28/II ANOVA TABLE OF MEANS BY CLONE BY TREATMENT, EXCLUDING PLANTS WHICH DID NOT FLOWER

	CLONE 6			CLONE 10		
	TRT. 1	TRT. 2	TRT. 3	TRT. 1	TRT. 2	TRT. 3
MEAN P/O PER FLOWER	20859	21142	67917	65515	29297	126953
MEAN VIABLE P/O PER FLOWER	43064	449144	317889	126033	51001	87195
TOTAL POLLEN/PLANT / TOTAL OVULE/PLANT	47.9	66.4	68.6	43.9	64.4	57.6
TOTAL VI. POLL./PLANT / TOTAL 'EFF.' OV./PLANT	15.8	14.4	25.5	12.7	25.9	14.2
MEAN G PER FLOWER	0.493	0.564	0.472	0.432	0.473	0.421
MEAN VIABLE G PER FLOWER	0.464	0.168	0.198	0.788	0.530	0.496

When discounting those plants which did not flower, a significant treatment effect is found for all variables. Plants subjected to treatments 1 and 2 exhibit significantly lower total and viable P/Os when compared with control plants. Plants subjected to treatment 1 have significantly lower total P/Os than those subjected to treatment 2. This increase in relative investment in female function under poorer conditions is also shown for mean viable G values. These are significantly higher for treatment 1 than for both treatment 2 and the control (treatment 3). However, a completely different pattern is revealed for mean 'total' G values. Treatment 2 gives a significantly higher mean total G (relatively more investment in female function) than either treatment 1 or the control, which are not significantly different. The explanation for this result is not apparent!

Overall, it would appear that these results are contradicting the theoretical predictions. Heslop-Harrison (1957) quotes some studies which, at first glance, appear to show similar results to those found here. (Molliard, 1897, and Joyet-Lavierge, 1931 quoted in Heslop-Harrison, 1957). However, Heslop-Harrison has shown other factors to be involved, and suggests that the results are not conclusive. It was assumed in the experiment described here that growing the plants in sand would simply decrease available nutrients. The quantities of the various nutrients available were not determined. Where previously mentioned papers have referred to rich or poor soils, they have

often quoted values for particular nutrients (e.g. nitrogen or potassium) only. Moisture content, which has also been shown to have an effect on relative investment in the male and female function (Freeman *et al*, 1980) was not determined. The sand may have been dryer than the soil in which the control plants were grown. The sand may also have caused some compaction of the underground parts of the plant due to its higher density compared with peat. Any one or more of these factors or others not mentioned may have affected the plant's allocation strategies.

Another possible explanation for the seemingly anomalous result of stress resulting in an increase in relative investment in female function is as follows. Solomon (1985), in his study on *Solanum carolinense*, found that plants grown in a medium containing high levels of fertilizer did not, as expected, tend more towards femaleness compared with control plants, but instead tended more towards maleness. He suggested that this was due to the high applications of fertilizer resulting in toxic levels of inorganic nutrients which stressed the plants and induced maleness. This situation of an excess of one or more factors causing the plant to become stressed, may be present in the experiment described here. Ideal growth conditions for *R. bulbosus* are not known. It was assumed that plants growing in peat were experiencing better conditions for growth than those grown in sand. However, the opposite may be true. Peat may provide an excess of some factor (perhaps inorganic nutrients or water) which place plants under stress. If this were the case,

theory would predict that plants grown in sand would be relatively more female than those grown in peat. This was the result of this experiment.

The inclusion of other floral structures in the measures of allocation, as discussed in the introduction to this chapter, may have resulted in different conclusions.

Although the actual strategy which has been revealed may contradict theory, it has been possible to show that *R. bulbosus* can alter its relative allocation to male and female gamete production under varying conditions. That is, it appears to exhibit ESD.

There is some evidence that taller standing flowers are functionally more female. This would appear to result from a decreased investment in pollen production, rather than increased investment in ovule production. It was originally hypothesised that pollen production may be higher in taller standing flowers because these would be more conspicuous to bees, resulting in a higher probability of pollen pick-up from such flowers. Perhaps this increased probability has an opposite effect, the greater efficiency resulting in lower pollen production. Since taller standing flowers do not exhibit higher levels of seed set or increased allocation to ovule production, it would seem unlikely that more resources are being allocated to seed production to take

advantage of the fact that taller flowers disperse seeds more widely (see Chapter 4).

3.3 GENERAL DISCUSSION OF THE CHAPTER

Initial observations on flowers collected from a field population of *R. bulbosus* at Rainbow Wood Meadow revealed variation between the flowers in anther:ovule ratio. This suggests that there may have been differences between plants, since each flower was collected from a different plant. However, the evidence for inter-plant differences was not conclusive. The experiments described in this chapter set out to prove more conclusively whether or not plants differ in their allocation to male and female function and, if so, how these differences come about. This included examining whether *R. bulbosus* exhibits environmental sex determination.

Lloyd's (1979, 1980) measure of effective gender (G) was used to measure the likely success of an individual as a mother or father within the study population. Bawa and Beach (1981) conclude that functional or effective gender is a more valuable estimate than those based on intrinsic gender or morphological appearance for illuminating variation in effective gender between conspecifics. Some studies have reported plants only as male, female or hermaphrodite (Bawa and Webb, 1983; McArthur, 1977; Schaffner, 1922; Schlessman, 1986). The studies involved here at least take into account actual gamete ratios and effective gender. P/Os have previously been used to quantify actual gamete ratios (Cruden, 1977; Cruden and Miller-Ward, 1981). They were used in the same way here.

Where possible, estimates of pollen viability were carried out. These demonstrated that a significant proportion of pollen may be inviable. The explanation for this is unclear. It may be that inviable pollen is cheaper to produce than viable pollen and is produced in order to maintain the attractiveness of the plant to pollinators. As this may assist the female as well as the male function, the production of inviable pollen cannot be viewed simply as an investment in male function. Until this matter can be resolved, caution must be exercised when investigating effective gender.

The experiments described in this chapter have explained some of the variation originally observed in the Rainbow Wood Meadow population. They have shown variation between both plants and flowers within plants.

Decreasing total allocation to gamete production per flower as the flowering season progressed was recorded. However, changes in relative allocation strategy were only exhibited by some of the plants. Therefore, some of the original differences observed between flowers in the Rainbow Wood Meadow population may have resulted from the fact that the sample did not contain equivalent flowers from the various plants in terms of number in the flowering sequence.

Although all flowers were collected from one field population of *R. bulbosus*, the genetic structure of this

population was not known. The plants from which the flowers were collected may have been genetically quite different. A genetic component to allocation strategy was revealed by the significant differences between clones, found in experiment b) and c).

Although ESD was not proven in all experiments, it would appear from experiment d) that it does have a rôle to play in the sexual allocation strategy of *R. bulbosus*. The field from which the flowers were collected contains different micro-habitats. For example, the field has a stream running through it (differences in soil moisture content), contains some trees and shrubs (differences in light intensity), and is grazed by a small herd of cattle which trample certain areas of the field more than others, and may fertilize areas on which they deposit their droppings. The field was also partly grazed by rabbits. These do not graze extensively on *R. bulbosus* (but see chapter 4), because it contains Ranunculin, but do graze on surrounding herbage, thus reducing competition in some areas. There are also many anthills in the field. Soil moisture, aeration and disturbance may be different on these. The selection of flowers from within the field was not made from only one particular habitat, so flowers are likely to have been collected from plants growing under varying conditions of soil, fertility, light intensity, moisture levels, competition and other factors. These different conditions may be causing differences in sexual allocation strategies.

ESD supposes that plants are somehow able to monitor their environment and use this information to alter their sexual allocation strategy. Charnov and Bull (1977) and Freeman *et al* (1980, 1981) suggest that plant hormones could be implicated in this. Freeman *et al* (1980, 1981) review evidence which shows that hormone levels vary under different conditions (e.g. water stress, salinity, and heat). They also discuss studies which show that application of hormones can alter the sexual allocation strategies of some plants.

The sexual allocation strategy adopted by a plant may be affected by the behaviour of its conspecifics. If prevailing conditions favour greater relative allocation to male gametes and ESD is operating, most plants are likely to be functionally more male. Competition between pollen from conspecifics to fertilize the available ovules will be great. A plant which, in such an area, allocates relatively more to female and less to male gametes than its conspecifics, may have a selective advantage. There will be no shortage of pollen to fertilize its ovules and by producing less pollen it will reduce competition with its conspecifics for mates. In such a situation there is likely to be a balance between the advantages and disadvantages of allocating more to the female function. This aspect was not investigated in this study.

CHAPTER 4

GENE FLOW IN A POPULATION OF *RANUNCULUS BULBOSUS* L. AT RAINBOW WOOD MEADOW, BATH

4.1 INTRODUCTION

4.1.1 GENE FLOW

Gene flow refers to the flow or migration of genes within and between populations. Levin and Kerster (1974) distinguish between 'potential' and 'actual' gene flow. 'Potential' gene flow is measured in terms of the movement of pollen and seeds. 'Actual' gene flow refers to the incidence of pollination and the establishment of reproductive individuals.

But 'what is a population?'. This is the very question asked by Crawford (1984 a). The answer depends upon the approach to the question. Genetic theorists have produced the concept of an idealised population in which mating occurs at random. Random mating is generally not the case in natural populations, and

departures from the idealised situation can be accommodated by using the effective population size (Ne). This is defined as "the number of breeding individuals in an idealized population which would show the same variance under random genetic drift, or the same amount of inbreeding, as the population under consideration" (Crawford 1984 a). Changes in the effective population size occur as a result of differences in the number of breeding individuals, unequal sex ratios, differential reproduction, inbreeding and overlapping generations (Cahalan, 1983).

Formulae for calculating, and the theory behind, Ne are given by Crawford (1984 a). However, there are practical difficulties in obtaining the measures required for its calculation (Cahalan, 1983). Because of these, many authors simply calculate the neighbourhood size or area. This is defined by Wright (1946) as "a unit within a continuous array of organisms from which the parents of an individual may be treated as if drawn from random". The neighbourhood size equals the effective population size only under certain conditions, but it is an indicator of Ne and it gives its upper limits (Cahalan, 1983).

Crawford (1984 a) discusses at length the details behind the calculations of neighbourhood sizes and gives examples in which he considers errors in calculation have been made. If, within a neighbourhood, individuals are considered to be distributed at uniform density, either linearly or areally, the length or area of the neighbourhood will depend upon the variance of

parent-offspring dispersal distance (σ^2) and the outcrossing rate (t). If dispersal distances are normally distributed the length (L) of a linear neighbourhood will be $2\pi\sigma$ and will contain 92.4% of the parents of a central individual. An areal neighbourhood will have a radius 2σ and an area (A) equal to $4\pi\sigma^2$ which will contain 86.5% of the parents of the central individual. The number of individuals composing a neighbourhood, the neighbourhood size, will also be dependent upon the effective density, usually estimated as the density of breeding individuals (d). Neighbourhood size is equal to $2\pi\sigma d$ for a linear neighbourhood, and $4\pi\sigma^2 d$ for an areal neighbourhood. In principle there will be a neighbourhood around any point in a population, the area of which depends upon the local biotic and abiotic conditions. These neighbourhoods will overlap. The estimate of neighbourhood area/size for a population is only an approximate estimate.

The estimate of neighbourhood given above assumes that dispersion variance is measured along a single axis and has a mean of Ø. It also assumes a normal distribution of dispersal distances. Any deviation from these assumptions must be corrected for in the calculation. Crawford (1984 a, b) deals with these problems. His corrected equations will be used to estimate neighbourhood size in a population of *R. bulbosus* at Rainbow Wood.

To measure neighbourhood size in plants it is necessary to obtain estimates of the variance of parent-offspring dispersal distances. One way of doing this is to measure the variance of

pollen and seed dispersal distances. This method was used during this research and it is, therefore, pertinent to consider it in some detail.

4.1.2 POLLEN DISPERSAL

In some plants, minimum pollen dispersal distances can be estimated because differences in morphology exist between pollen from different plants (e.g. pin and thrum plants in *Primula vulgaris*; see Cahalan, 1983). However, where this is not the case other methods of tracking pollen have to be employed. These include the use of fluorescent dyes (Linhart, 1975; Stern and Mueller, 1968; Stockhouse, 1976) and of radioisotopes (Colwell, 1951; Reinke and Bloom, 1979; Schlising and Turpin, 1971; Turpin and Schlising, 1971) and the use of neutron activation analysis (Fendrik and Glutrecht, 1967; Gaudreau and Hardin, 1974; Handel, 1976). They also include following pollinators as they forage. This last method was the one used during this research.

The efficiency of dispersal method will limit the success of pollination (Alexander, 1987). *R. bulbosus* pollen is dispersed by animals (zoophilous dispersal). In the case of zoophilous plants, patterns of pollen dispersal will be determined by the foraging behaviour of the pollinator. Some authors use this behaviour (e.g. flight distances) as a direct measure of pollen flow (see Levin, 1981, for references).

It must be remembered that pollinators do not consciously set out to pollinate plants but rather 'accidentally' effect pollination as a result of their foraging. Pollinators will forage in such a way as to maximize their energy gain (Pyke, 1978 b). Therefore, plants have evolved in such a way as to try to maximize the amount of pollen transferred to conspecific stigmas via pollinating agents.

A large body of literature has been produced over the years to investigate the effects of foraging behaviour on gene flow and to see how various plant properties can affect this behaviour. Much of the work has been conducted using bees as pollinators.

A major effect that has been investigated is that of plant spacing on levels of gene flow. Bees tend to fly between plant nearest neighbours. This is reflected in the inverse relationship between bee-flight distance and plant density (Beattie, 1976; Campbell, 1985; Levin and Kerster, 1969 a, b; Levin and Kerster, 1974; Schaal, 1978). Because foraging pattern is density dependent, so is neighbourhood size/area (Levin and Kerster, 1968; Schaal, 1978). It also results in a leptokurtic distribution of pollinator flight distances (Bateman, 1950; Crawford, 1984 a; Levin and Kerster, 1968; Levin and Kerster, 1969 a).

Levin and Kerster (1969 b) suggest that this pattern of foraging behaviour makes it possible to measure gene flow directly from plant spacing. However, Levin (1981) later suggested that

data calculated in this way may give an underestimate of the extent of gene flow.

One reason for this is the occurence of pollen carryover. Several authors have shown that all pollen may not be deposited on the first flower visited after pollen pick up. Instead, it may be transported to the second, third, fourth and even up to the 54th flower in a sequence of visits (Campbell, 1985; Levin, 1981; Levin and Kerster, 1974; Price and Waser, 1982; Thomson, 1986; Thomson and Plowright, 1980). Generally there is an exponential decrease in pollen deposition as a function of flower number in the sequence (Campbell, 1985; Thomson, 1986; Thomson and Plowright, 1980).

A number of factors have been shown to affect the amount of carryover. These include resource quality, for example, nectar production (Heinrich, 1979; Pleasants and Zimmerman, 1979; Thomson, 1976; Thomson and Plowright, 1980; Waddington, 1981; Willson, 1984; Zimmerman, 1982 b) and pollen quality (Percival, 1955). Increasing nectar quantity and quality can increase the time spent by the pollinator in each flower and consequently the amount of pollen deposited in a flower (Levin, 1981; Oshawa and Namai, 1988; Thomson, 1986; Thomson and Plowright, 1980). This will leave less pollen to be deposited in subsequent flowers.

Heinrich (1979), Pleasants and Zimmerman (1979) and Zimmerman (1982 b) report the presence of 'patches' of different resource quality with respect to nectar, in arrays of plants. The behaviour of pollinators in 'good' and 'poor' patches differs. Pollinators move quickly through 'poor' patches and concentrate on 'good' ones, visiting more florets per head in these areas than in poor ones. The average flight distance in 'poor' patches is higher than in 'good' patches.

The effect of pollen carryover may be increased if there is directionality of flights (Heinrich, 1979; Levin *et al*, 1971). Pleasants and Zimmerman (1979) have shown patch type to have an affect on directionality, with the angle of departure increasing with the value of the reward. Bees thus tend to stay in a 'good' patch. However, Zimmerman, (1982 a) studying *Bombus bifarius* and *B. flavifrons* foraging on *Potentilla gracilis* has shown that pollinators move at random with respect to directionality. Zimmerman suggests that this behaviour will be adaptive when there is no penalty associated with a return to a foraging area.

Different pollinators may show different behaviour (Herrera, 1987 a, b; Levin and Kerster, 1974; Morse, 1978, 1982; Primack and Silander, 1975; Schmitt, 1980; Zimmerman, 1982 b) and/or morphology (Inouye, 1980). These may affect pollen dispersal. For example, *Lepidoptera* fly longer distances between plants, visit fewer flowers per head and fewer plants per feeding bout than *Hymenoptera* (Herrera, 1987a, b; Scmitt, 1980). Schmitt

(1980) calculates neighbourhood size using pollen dispersal variances measured for butterflies and bees separately. Neighbourhood sizes for butterfly-pollinated plants are much larger than for bee-pollinated plants (993 - 6,154 versus 8 - 25 respectively) and a small amount of butterfly pollination in a predominantly bee pollinated population can greatly increase neighbourhood size.

4.1.3 SEED DISPERSAL

Variance of seed dispersal is the other major component required for estimating neighbourhood size in seed plants. Slatkin (1985 a) suggests that seed dispersal has much greater potential than pollen as a source of gene flow. Antonovics (1968) reaches the opposite conclusion, however. Many estimates of seed dispersal show that most seed is dispersed close to the source (Herrera, 1987 b; Levin, 1981; Levin and Kerster, 1968; Levin and Kerster, 1974). This results in a leptokurtic dispersal distribution. However, Levin (1981) suggests that many seed dispersal estimates may be too conservative because it is difficult to measure long distance dispersal. Slatkin (1985 a) argues that the speed with which many plants colonize vacant habitats implies long distance gene flow via seeds. However, Levin and Kerster (1974), although agreeing that species extension is decided by long distance dispersal, conclude that the effect of seed production on the breeding structure of a species is decided

by the relationship between local seed dispersal and that at longer distances.

Levin and Kerster (1974) report that, in general, high seed dispersal is favoured in a varying environment and seed size increases as the environment becomes more reliable.

Seed dispersal estimates are generally measured in terms of absolute distances from seed source. However, Levin (1981) suggests that a better way of measuring dispersal would be in terms of the number of juxtaposed plant canopies over which the seed disperses. This is because the size of the plants varies so much. A dispersal distance of 25 m would be a long way to a small herbaceous plant but a relatively shorter distance for a large tree. Using data on *Phlox drumondii* and *Eucalyptus regnans* he shows that seed dispersal distances of 1.2 m - 3.6 m and 40 m - 125 m respectively convert into 4 - 10 plant diameters and 5 - 15 plant diameters respectively. This shows that, although absolute dispersal distances in *Eucalyptus* may appear much larger than in *Phlox*, they are very similar in terms of the number of conspecifics dispersed over.

Seed dispersal may be achieved by three main vectors: animal, wind, or water. Most of the definitive evidence on seed and fruit dispersal relates to wind dispersed species (Levin and Kerster, 1974). Dispersal distances in wind dispersed seeds depend primarily upon settling rate, height, and area of the seed

source, turbulence and wind velocity. Wind dispersed seeds have adapted structures and mechanisms to increase dispersal distances; for example, increasing the relative height of dispersal by having explosive mechanisms of dispersal (*Phlox pilosa*, Levin and Kerster, 1968).

Seeds dispersed by animal vectors may also show adaptations, but of a different kind. They may have hairs or barbs to catch in the covering of animals. They may have fleshy fruit to attract animals to eat the seeds, which then pass through the gut unharmed and are deposited where the animal defaecates. They may also have large seeds which animals collect and store at distances away from the source. Plants with animal dispersed seeds should synchronize seed production with greatest dispersal agent activity.

Predator behaviour may influence seed dispersal. For instance, many predators forage in a density-dependent manner, increasing foraging in areas of high seed density. They may also forage more voraciously closer to the parent plant (Janzen, 1970, 1971, 1972, from Levin and Kerster, 1974).

4.1.4 POTENTIAL *VERSUS* ACTUAL GENE FLOW

Measures of pollen and seed dispersal distance give estimates of potential gene flow. However, dispersal does not guarantee gene flow (Levin, 1981). Levin (1981) suggests that measures of potential gene flow may be underestimates of actual

gene flow and, therefore, underestimate neighbourhood size. He discusses how such discrepancies may occur as a result of variation in pollen-pistil compatibility, seed viability, seed set, fitness of offspring, or mate selection. This area has generated a massive amount of literature. Some of the most important findings are summarised below.

i) With restricted gene flow, neighbouring plants are likely to be related. These are likely to share the same incompatibility alleles (Levin, 1981; Willson, 1984; Winsor et al, 1987) when incompatibility is present. This will reduce the efficiency of local pollen dispersal, increase that of distant pollen dispersal, and consequently lead to an increase in the ratio of actual to potential gene flow (e.g. Levin, 1981 on *Phlox drumondii*).

ii) Mating between neighbours which are related can lead to inbreeding depression and loss of heterosis (Banyard and James, 1979; Ellstrand et al, 1978; Schaal and Levin, 1981). The fittest progeny may result from distant pollen dispersal. However, there may be an optimal outcrossing distance as favourable gene combinations may be disrupted following crosses between widely separated parents, leading to outbreeding depression (Hughes and Vickery, 1974; Kruckeberg, 1957; Moll et al, 1965; Price and Waser, 1979; Willson, 1984). This effect may be magnified when there is already restricted gene flow. Interestingly, because of conflicting selection pressures on the plant and its pollinator, the optimum outcrossing distance may not

always equal the pollen flow distance (Price and Waser, 1979; Waddington, 1983). Thus, because of outbreeding depression, long distance pollen flow may have less affect on actual gene flow than that predicted from a simple measure of its incidence.

iii) Willson (Willson, 1979; Willson and Burley, 1983) has argued that sexual selection may operate. This may occur at the pollination stage (e.g. Janzen, 1977; Marshall and Ellstrand, 1985; Wade and Arnold, 1980), during growth of the pollen tube (Ennos and Dodson, 1987; Epperson and Clegg, 1987; Marshall and Ellstrand, 1985) or after fertilization, through selective abortion (Janzen, 1977; Moran and Brown, 1980; Nakamura and Stanton, 1987; Winsor *et al*, 1987). However, selective abortion may result from parent-offspring conflict rather than from sexual selection (Mazer, 1987).

These findings show that pollen reaching a conspecific stigma may not fertilize an ovule. Even if fertilization does occur, the resulting seed may abort or the seedling may be outcompeted by other, more vigorous seedlings. Potential gene flow, therefore, gives only an estimate of actual gene flow although the two are correlated (Govindaraju, 1988).

Actual gene flow may be more accurately measured by following the movement of genetic markers through a population (Cahalan, 1983; Rai and Jain, 1982; Smyth and Hamrich, 1987). These are often dominant alleles which can be detected through

observing phenotype. Schaal (1980) used a genetic marker to measure the pollen flow component of gene flow in *Lupinus texensis* and compared this with measures of pollinator flight distance. She reports pollen flow distances of 1.82 $\pm$ 0.10 m using the genetic marker technique and 0.97 $\pm$ 0.08 m using pollinator flight distances.

The measurement of actual gene flow has been aided by two developments; protein electrophoresis to detect alleles and more elaborate statistical methods (Lewontin, 1973, 1985). This has enabled plant geneticists to use marker genes and statistical models to describe plant mating patterns (Clegg, 1980).

These two developments have made it possible to look at the results of matings and reason backwards to original events using a model as a reference point. This is particularly useful in plants because the family unit is easily sampled (Clegg, 1980). There are several ways of studying the observed pattern of genes in a population. It can involve looking at the frequency of lethal alleles in the population, calculating the inbreeding coefficient or a related quantity, studying the distribution of rare alleles in the population or calculating genetic distances. All these methods are described by Slatkin (1985 a). These parameters can all be applied to whole populations (to study differences between populations) or to sub-sets of populations (to see if there is heterogeneity of gene flow in a population).

A number of authors have used these types of method to estimate gene flow. Bos *et al* (1986) used poly-acrylamide gel electrophoresis to look at allozyme variation at eight loci in *Plantago lanceolata*. They calculated genetic distances and fixation indices within and between populations. They found substructuring in populations and deviations from Hardy-Weinberg expectations which suggest localized gene flow. Ellstrand *et al* (1978) used allozyme data to calculate the degree of apparent outcrossing and fixation indices for populations of *Helianthus annuus* at varying densities. They found increasing density caused decreasing outcrossing. This was probably due to reductions in gene flow. They also found heterozygote deficiency in all populations, which is consistent with restricted gene flow. Other examples using these types of methods can be found in Golenberg (1987), Govindaraju (1988), Kirkpatrick and Wilson (1988), Shen *et al* (1981) and Slatkin (1981 and 1985 b).

The technique of electrophoresis does have its limitations in that it may not uncover all alleles at a locus in a population. Some alleles may only be detected if the conditions of electrophoresis (e.g. pH, voltage, and duration of run) are changed (Cahalan, 1983). Lewontin (1985) reports on cases where 'new' alleles have been discovered by changing conditions. The over-representation of highly variable structural and broadly specific enzymes in many electrophoretic studies may result in an over-estimate of polymorphism (Cahalan, 1983). Recent advances in DNA studies (e.g. DNA sequencing) may provide a more sensitive

method for examining genetic variation in natural populations and may have a rôle to play in future developments (Lewontin, 1985).

There are inherent difficulties associated with attempts to estimate gene flow levels in a population from parent-offspring dispersal and from patterns of genetic variation. Estimates obtained from parent-offspring dispersal distances fail to take into account such things as catastrophic events in the past, or long-term fluctuations, since they deal only with the standing crop. They are short-term estimates. Levin and Kerster (1968, 1974) suggest that using levels of genetic variation to measure population size presents a circular argument. In doing so, the very effects that are induced by finite population are used to measure that size. Calculating neighbourhood sizes by these two types of method may, therefore, give very different results.

Neighbourhood sizes have been measured (by both methods) in a number of species. Some results are published in Bos *et al* (1986), Cahalan (1983), Cahalan and Gliddon (1985), Govindaraju (1988 b), Levin (1981), and Levin and Kerster (1968, 1969 a and 1974). It has generally been found that neighbourhood size is restricted, although this is not always the case (e.g. Govindaraju, 1988 b).

4.1.5 THE SIGNIFICANCE OF GENE FLOW

Levels of gene flow have been of interest to plant breeders and seed producers for a long time (e.g. Bateman, 1947). With the development of new improved strains of crop plants, for instance, it has become necessary to ensure genetic purity of seeds by preventing inbreeding. Only relatively recently has the subject of gene flow become of interest to evolutionary biologists, the scale of gene flow (estimated by neighbourhood size, for instance) being critical to the pattern of genetic substructuring in the population (Antonovics, 1968; Ellstrand and Marshall, 1985; Schaal, 1978; Slatkin, 1985 a).

Restricted gene flow may lead to inbreeding depression, loss of heterozygosity or random changes in gene frequency (Lynch, 1986; Lewontin, 1973). It may also enable organisms to respond to localized selective forces and thus adapt to the environment.

There is still some debate as to the extent of gene flow within and between populations (see Cahalan, 1983, for discussion). It was originally thought to be extensive, but a recent body of literature suggests that it may be more restricted than was thought (Beattie, 1976; Bos *et al*, 1986; Brown, 1979; Ehrlich and Raven, 1969; Golenberg, 1987; Levin and Kerster, 1968, 1974; Rai and Jain, 1982; Schaal, 1980). Ehrlich and Raven (1969) conclude from this that the sub-population (effective population or neighbourhood) is the unit of evolution

rather than the species, i.e. they take the group selectionist view.

Wright (1946) suggests that the extent of local differentiation due to drift (rather than selection) may be predicted from the estimated neighbourhood size. A neighbourhood size of: $\leqslant$ 20 will show considerable drift; $\leqslant$ 200 will possibly exhibit drift; $\geqslant$ 1,000 will be virtually panmictic and drift will be improbable. A number of authors have estimated neighbourhood sizes which are, by Wright's predictions, small enough to allow differentiation due to drift (Bos *et al*, 1986; Bos and van der Haring, 1988; Cahalan, 1983; Slatkin, 1981 and 1985 a).

Prevention of divergence of local populations due to drift requires sufficient gene flow, or the operation of similar selective forces such as stabilizing selection between the various sub-populations (Lynch, 1986). However, the environment occupied by a population is unlikely to be homogeneous in its selective pressures (Antonovics, 1968) and the reactions of plants to differences in the environment may, in itself, lead to differentiation. This has been found to be the case in a number of studies (Hamrick and Holden, 1979; Lande, 1976; Linhart *et al*, 1981; Schaal, 1984; Speiss, 1968; Turresson, 1922). Limited gene flow will be important here (Brown, 1979), with the pattern of differentiation being determined by the interaction between selection and gene flow (Antonovics, 1971; Felsenstein, 1976; Jain and Bradshaw, 1966; Levin and Kerster,1968; Rai and Jain, 1982;

Slatkin, 1985 a). Levin and Kerster (1974) conclude that "most species of seed plants will be composed of multiple isolated and semi-isolated breeding units of varying size and area, each adapted to local environmental conditions".

Where selective pressures are high and/or gene flow levels very low, differentiation may occur over very short distances. An example of this is differentiation between groups of plants growing on heavy-metal-contaminated slag heaps and those in surrounding fields. The plants growing on the slag heaps have a high selective pressure on them for heavy metal tolerance, which those in surrounding areas do not. (Allard *et al*, 1972; Antonovics, 1971; Jain and Bradshaw, 1966). Where selective pressures are lower and/or gene flow levels higher, a clinal pattern of differentiation would be expected, with the correlation in gene frequencies between neighbourhoods decreasing with distance (Levin and Kerster, 1968; Pamilo, 1988; Slatkin, 1985 a). The important factor determining the relatedness of neighbourhoods is, in fact, the number of neighbourhoods between the two in question rather than the absolute distance (Cahalan, 1983; Cahalan and Gliddon, 1985).

So the pattern of genetic variation in plant species may be complex, consisting of clines interspersed with sharper changes in gene frequencies. This has been referred to as a 'graded patchwork' by Jain and Bradshaw (1966).

Temporal variation in environment may also be important in determining the pattern of variation in species. More constant environments will exhibit larger neighbourhood sizes. This is due to the average neighbourhood size in time being the harmonic mean of neighbourhood sizes. The harmonic mean is biased towards smaller values.

Adaptations to local environment can be advantageous in more ways than one. The variation in gene frequencies between populations will maintain genetic variability within the species as a whole (Felsenstein, 1976; Levin and Kerster, 1974) It will also allow different members of the same species to occupy a variety of niches, thereby increasing the overall range and productivity of the species (Antonovics, 1971; Jain and Bradshaw, 1966; Levin and Kerster, 1974). Fixation of advantageous genes in sub-populations, resulting from low levels of gene flow interspersed by long-distance dispersal events, may help in the spread of advantageous genes through the population (Slatkin, 1985 a).

It is difficult to assess the relative importance of localized selection and drift, especially when patches are variable (Willson, 1984). However, it will generally be the case that differentiation due to random drift will be more important when the neighbourhood size is small compared with the size of environmental patches over which selection acts uniformly (Cahalan and Gliddon, 1985).

Local differentiation due to restricted population size can have both advantageous and deleterious effects. Part of the aim of this project is to estimate neighbourhood size in a population of *R. bulbosus* at Rainbow Wood. This will be achieved by measuring pollen and seed dispersal variances together with flowering density.

4.2 EXPERIMENTAL AND RESULTS

In this section, the general methods used will first be described. Each experiment performed will then be outlined and the results presented. A short discussion will accompany each set of results. A further discussion, relating these results to those in the literature, will be presented in the next section.

4.2.1 GENERAL METHODS

(Hand Pollination and Seed Germination)

a) Bagging of flowers

The source and supply of pollen had to be controlled in the pollination experiments. In order to exclude unwanted pollen, it was necessary to create a barrier between the flower being pollinated and any external pollen vectors. To achieve this, flowers were covered with muslin bags just before opening and re-covered immediately after any treatments had been applied. The muslin bags were tied around the flower stem just tightly enough to stop insects from entering but not so tight that the flower was killed. Muslin was used in preference to plastic to allow free flow of air whilst excluding pollen. This prevents the build-up of heat and noxious gases, which may affect pollen and ovule viability. Bags were left in place until six weeks after flowering, by which time seed had matured.

b) Hand pollination of flowers

To effect crosses between particular parents it was necessary to control the movement of pollen from one flower to another. This was achieved by hand-pollinating flowers. Pollen from the intended male parent/s was collected on a small paintbrush which was then brushed over the stigmas of the intended female parent. The paintbrush was dipped in alcohol and allowed to dry between each cross. This avoided contaminating the stigma with pollen carried over from previous pollinators.

c) Emasculation of flowers

To prevent contamination of an hermaphrodite flower with self-pollen, it was necessary to remove all pollen bearing structures from the flower before pollen release. This was achieved by simply nipping out all of the anthers before anthesis, using a pair of fine forceps.

d) Seed germination

Seeds were collected whilst still slightly green (approximately six weeks after pollination) and stored in a desiccator at room temperature. It is important to store seeds in dry conditions in order to prevent loss of viability. Seeds stored for a long time, even under dry conditions, showed loss of viability.

Seeds were planted in trays filled with a mixture of Fison's M2 compost and fine sand (1:1 ratio). Following the guidelines of Harper (1957), seeds were planted at a depth of 1.6 cm. At the two-leaf stage seedlings were planted singly into 3 " pots filled with Fison's M2 compost and left in an unheated greenhouse. Once established, the plants were moved outside and left until required.

4.2.2 MEASUREMENT OF NEIGHBOURHOOD SIZE

a) Introduction

The theory of gene flow was discussed in detail in the main introduction. The aim of this project was to estimate neighbourhood size in a population of *R. bulbosus L.* at Rainbow Wood. This was achieved by measuring pollen and seed dispersal variances, density of reproductive individuals and outcrossing rate.

Pollen dispersal variance was estimated using the variance of bee flight distances in the field, together with measurements of pollen carryover. The principle behind the measurement of pollen carryover is that *R. bulbosus* is self-sterile. Therefore, although a bee foraging among a group of cloned plants of the same genotype will transfer pollen between the plants, this pollen will not effect fertilization. If, before it visits a clone being used as a pollen recipient, a bee first picks up pollen from a plant of a different genotype to that of the clone, it will deposit this pollen on the stigmas of the clone and effect fertilization. Determination of seed set in the pollen recipient will allow pollen carryover to be measured. Since plants are self-sterile, any seed set must result from pollen from the single plant of a different clone being deposited on the stigmas and fertilizing the ovules of the group of cloned plants (if all other pollen is excluded).

As far as is known, *R. bulbosus* seeds have not been reported as exhibiting structures to aid long-distance dispersal by animal or other vectors. Therefore, only local seed dispersal was measured. This may result in underestimates of seed dispersal variances.

Outcrossing rate ($\underline{t}$) varies from 0 to 1, 0 representing complete inbreeding, 1 complete outbreeding. Only rarely will estimates of $\underline{t}$ be exactly equal to 1, although they may approach 1 in completely self-sterile individuals. There has been some debate as to the extent of selfing in *R. bulbosus* (see Chapter 1 for discussion).

Before trying to measure exact levels of outcrossing, it was decided to determine if *R. bulbosus* was self-sterile. If this was the case, it would be reasonable to estimate $\underline{t}$ as 1.

b) Experimental

1) An experiment to determine whether *R. bulbosus* is self-sterile

i) Methods

This experiment was initiated on 3/4/86, when ten plants of different genotypes were selected from greenhouse stock. Each plant was placed in a different room to reduce the risk of uncontrolled cross-pollination between plants. The plants were visited each day and any nearly open flower buds (see Fig. 3.1) were bagged with muslin. Once open, flowers were treated in one of five ways:

a) Left bagged until seed set.
b) Hand-pollination was carried out within the same flower.
c) Hand-pollination was carried out between different flowers on the same plant.
d) Hand-pollination was carried out between flowers on different plants using plants as both males and females. Reciprocal crosses were not carried out between pairs of flowers, since flowers acting as male parents were removed from the plant and carried to the female parent where the cross was made.
e) Emasculation.

All flowers were bagged immediately after treatment.

Treatment c) was carried out because anthers and carpels in the same flower may not have been mature at the time a 'within-flower' cross was made. Crossing between flowers within a plant ensured that mature stigmas were pollinated with mature pollen.

Cross-pollination was carried out between flowers of different plants to ensure that bagging the flowers had no adverse effects on pollen and ovule viability, since seed set should occur in most out-crosses.

Flowers were emasculated as a control to check that contamination with foreign pollen was not occurring when not required.

The muslin bags were removed approximately six weeks after flowering and the percentage seed set for each flower was determined.

ii) Results

The results are contained in tables 4.1, 4.2, and 4.3. None of the flowers which were selfed or emasculated set seed. Most outcrossed flowers set seed at a rate of between 23% to 95%. However, not all outcrosses produced seeds. Where seed was not set, the carpels had sometimes swelled in size compared with those in emasculated flowers. When examined further, these swollen

TABLE 4.1 NUMBER OF FLOWERS IN WHICH SEED SET

FOR THE FIVE TREATMENTS

	TREATMENT				
	EMASCULATED	LEFT BAGGED	HAND-POLLN WITHIN FLOWER	HAND-POLLN BETWEEN FLOWERS	OUTCROSSED
NUMBER OF FLOWERS IN WHICH SEED SET	0	0	0	0	48
TOTAL NUMBER OF FLOWERS	40	179	55	82	73
PERCENTAGE OF FLOWERS IN WHICH SEED SET	0	0	0	0	65.75

TABLE 4.2

MEAN SEED SET FOR OUTCROSSED FLOWERS

MALE PLANT	MEAN SEED SET PER PLANT ACTING AS FEMALE (%)									
	PLANT 1	PLANT 2	PLANT 3	PLANT 4	PLANT 5	PLANT 6	PLANT 7	PLANT 8	PLANT 9	PLANT 10
1	-	63.00	33.33	51.22*	ND	ND	40.38	14.07	0	66.67*
2	35.8	-	ND	28.57*	ND	87.5*	82.76*	25.00	66.35	83.72*
3	0*	ND	-	ND	ND	ND	ND	ND	ND	ND
4	34.21*	53.22	0*	-	ND	ND	ND	ND	ND	66.67*
5	0*	ND	ND	ND	-	ND	ND	ND	ND	62.79*
6	ND	0*	ND	ND	ND	-	ND	ND	ND	ND
7	69.23*	76.19*	ND	34.21*	83.50*	ND	-	25.80	ND	45.55
8	0*	ND	17.54	ND	ND	ND	ND	-	43.75	60.62
9	12.76	ND	68.86	37.50*	0*	ND	33.33*	ND	-	0*
10	0*	ND	ND	ND	ND	ND	ND	ND	0	-

ND = No Data

* = Only 1 value for seed set

TABLE 4.3

ACTUAL SEED SET FOR OUTCROSSED FLOWERS

DATE	MALE PLANT	FEMALE PLANT	% SEED SET	DATE	MALE PLANT	FEMALE PLANT	% SEED SET
14/4/86	1	2	62.79	29/4/86	4	2	67.86
21/4/86	1	2	39.53	1/5/86	4	3	0
24/4/86	1	2	58.97	13/5/86	4	10	66.67
29/4/86	1	2	63.89				
29/4/86	1	2	55.55	12/5/86	5	1	0
29/4/86	1	2	65.79	6/5/86	5	10	62.79
2/5/86	1	2	66.67				
6/5/86	1	2	84.85	25/4/86	6	2	0
8/5/86	1	2	68.96				
14/4/86	1	3	66.67	28/4/86	7	1	69.23
8/5/86	1	3	0	30/4/86	7	2	76.19
21/4/86	1	4	51.22	28/4/86	7	4	34.21
24/4/86	1	7	80.77	30/4/86	7	5	85.50
2/5/86	1	7	0	30/4/86	7	8	0
28/4/86	1	8	0	9/5/86	7	8	51.61
6/5/86	1	8	0	2/5/86	7	10	0
12/5/86	1	8	42.22	6/5/86	7	10	91.11
28/4/86	1	9	0				
8/5/86	1	9	0	6/5/86	8	1	0
5/5/86	1	10	66.67	28/4/86	8	3	12.0
				13/5/86	8	3	23.08
23/4/86	2	1	32.50	28/4/86	8	9	87.50
29/4/86	2	1	75.00	15/5/86	8	9	0
6/5/86	2	1	0	28/4/86	8	10	83.33
22/4/86	2	4	28.57	6/5/86	8	10	0
29/4/86	2	6	87.50	13/5/86	8	10	92.50
14/4/86	2	7	82.76	15/5/86	8	10	66.67
29/4/86	2	8	50.00				
6/5/86	2	8	0	28/4/86	9	1	25.53
22/4/86	2	9	95.45	7/5/86	9	1	0
24/4/86	2	9	87.80	24/4/86	9	3	42.86
28/4/86	2	9	66.67	28/4/86	9	3	94.87
29/4/86	2	9	81.82	24/4/86	9	4	37.5
8/5/86	2	9	0	22/4/86	9	5	0
6/5/86	2	10	83.72	28/4/86	9	7	33.33
				15/5/86	9	10	0
19/5/86	3	1	0				
				16/5/86	10	1	0
29/4/86	4	1	34.21	2/5/86	10	9	0
22/4/86	4	2	38.30	19/5/86	10	9	0
22/4/86	4	2	53.49	19/5/86	10	9	0

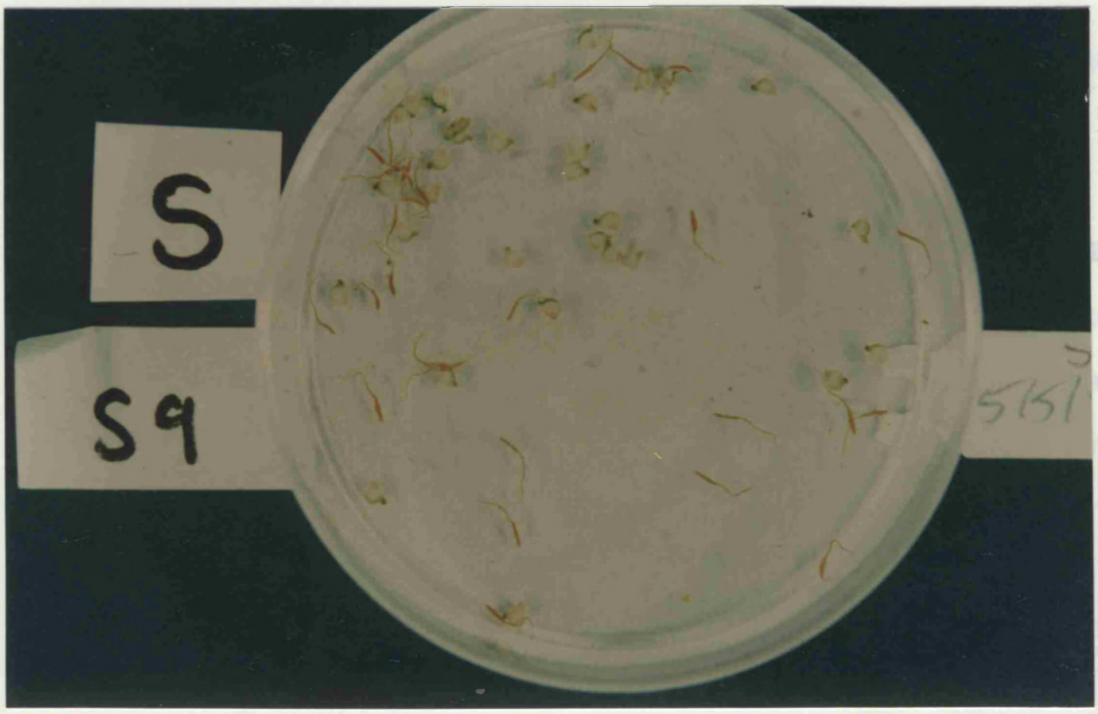


fig 4.1 Unfertilized carpels collected six weeks after pollination (not swollen)

Fig 4.2 Unfertilized carpels collected six weeks after pollination (slightly swollen)

carpels contained no embryo. Some carpels in selfed flowers also exhibited this phenomenon (see Figs. 4.1 and 4.2).

It can be concluded that out of a possible 14,813 carpels which could have set seed (350 flowers, average number of carpels/flower = 41.61 gives 14,813 carpels in total), no seed was produced by self-pollination. It therefore seems reasonable to assume that *R. bulbosus* is an obligate outcrosser.

iii) Discussion

There were two observations from this experiment which warranted further investigation. These were the enlarged carpels found in some outcrossed and selfed flowers and the variation in seed set between outcrossed flowers.

No enlarged carpels were noted in emasculated flowers, which suggested that stimulation by pollen is required in their formation. The enlarged carpels may give some clues as to the timing of the self-incompatibility (SI) mechanism in *R. bulbosus*. A late acting SI mechanism (e.g. at the stage of pollen-tube penetration of the ovule) may result in some enlarging of the carpels. An earlier acting SI mechanism (e.g. prevention of pollen-tube growth) may result in no enlargement of the carpels. This was investigated further during the next flowering season.

There was no pattern to the variation in seed set between outcrossed flowers. The variation may have resulted from environmental factors. The plants were kept in widely separated rooms to avoid accidental cross-pollination. Also, the temperature rose between April and May. Either or both of these factors may have had an effect on rate of the seed set. Some of the plants may have been close relatives. This would increase the chance of shared incompatibility alleles and may result in reduced seed set. This is unlikely since the plants were collected from various widely separated sites around the University of Bath. Also, if incompatibility alleles were involved, it would be more likely for a pattern to emerge in the results of crosses.

The rôle of environmental effects in the variation of seed set was further investigated during the next flowering season.

2) An experiment to investigate some of the unusual observations from experiment 1

i) Methods

To investigate the differences in percentage seed set, eight of the plants from Experiment 1 (two had died) were cloned to produce at least ten plants of each clone type. When plants commenced flowering, one plant of each clone was placed in one of five different sites. These were window sills facing north, south, east and west in the Biology building and a greenhouse. The sites were chosen to be representative of the positions of plants in Experiment 1. Cross-pollinations were then carried out between plants according to table 4.4. The crosses performed were dependent upon which plants flowered in unison. Crosses were kept standard at all five sites.

Flowers were crossed on the first day of opening, and thereafter on two successive days. After each cross, the flowers were bagged and labelled with the dates of the three crosses. Some flowers were left uncrossed, bagged, and labelled with the date of flower opening. This was to check that no self-fertilization was occurring. As many crosses as there were flowers available were performed throughout the flowering season. Seed heads were collected at the end of June, and percentage seed set for each cross was calculated.

TABLE 4.4

CROSSES CARRIED OUT AT EACH SITE

	1	2	8	9
1		✓	✓	✓
2	✓		✓	✓
8	✓	✓		✓
9	✓	✓	✓	

	3	4	5	10
3		✓	✓	✓
4	✓		✓	✓
5	✓	✓		✓
10	✓	✓	✓	

Statistically significant differences in seed set between rooms, between plants when acting as pollen recipients (females) and between plants when acting as pollen donors (males), were investigated using a one-way analysis of variance (Minitab statistical package). The statistical package used also provides an unplanned comparison between means to show which means are significantly different.

In order to investigate the phenomenon of swollen carpels, five plants, each of a different genotype, were selected from the clones of the eight plants left from Experiment 1. Approximately half of the flowers produced by each plant were castrated before opening, then immediately bagged and labelled. The other half were bagged prior to opening, and once open were self-pollinated, bagged and labelled.

All seed heads were collected at the end of June, 1988. This meant that different seed heads had been left on the plants for different lengths of time. Ages ranged from 65 days to 85 days after flower opening. The seed heads were examined for any signs of swollen carpels.

ii) Results

The summarised results of outcrosses are contained in tables 4.5, 4.6, and 4.7. A significant difference in percentage seed set was found between rooms. Plants in the east-facing room and

TABLE 4.5

TABLE OF MEAN SEED SET PER ROOM

ROOM NUMBER	TOTAL NUMBER OF CROSSES	MEAN SEED SET (%)	STANDARD DEVIATION
1 (North facing)	50	38.43^{ab}	37.02
2 (East facing)	72	25.33^{b}	32.13
3 (South facing)	71	36.95^{ab}	28.21
4 (Greenhouse)	76	30.17^{b}	29.05
5 (West facing)	130	45.49^{a}	29.66

$F(4,394) = 6.00$ ($p < 0.005$)

Superscripts denote which means were found to be significantly different from one another using an unplanned comparison of the means.

TABLE 4.6

TABLE OF MEAN SEED SET PER PLANT
FOR PLANTS ACTING AS FEMALES
(I.E. POLLEN RECIPIENTS)

PLANT CLONE TYPE NUMBER	TOTAL NUMBER OF CROSSES	MEAN SEED SET (%)	STANDARD DEVIATION
1	53	36.18[a]	37.48
2	40	48.54[a]	30.63
8	55	33.49[a]	18.55
9	48	36.75[a]	36.85
3	49	45.13[a]	24.28
4	49	15.70[b]	19.22
5	50	31.38[a b]	33.88
10	52	45.36[a]	34.52

$F(7,388) = 5.70$ ($p < 0.005$)

Superscripts denote which means were found to be significantly different from one another using an unplanned comparison of the means.

TABLE 4.7

TABLE OF MEAN SEED SET PER PLANT
FOR PLANTS ACTING AS MALES
(I.E. POLLEN DONORS)

PLANT CLONE TYPE NUMBER	TOTAL NUMBER OF CROSSES	MEAN SEED SET (%)	STANDARD DEVIATION
1	42	34.64	31.69
2	43	33.52	31.59
8	56	42.54	36.74
9	49	32.68	28.47
3	46	33.95	31.49
4	48	33.78	32.12
5	52	36.29	31.00
10	54	34.03	30.77

$F(7,382) = 0.53$ ($p > 0.05$)

the greenhouse set significantly less seed than plants kept in the west-facing room. Plants of clone type 4 set no seed in the east- or north-facing rooms. Plants of clone types 5 and 9 set no seed in the east-facing room. Clone type 10 set no seed in the greenhouse.

A significant difference in percentage seed set was also found between plant clone types acting as pollen recipients (females) but not between plant clone types acting as pollen donors (males). Plants of clone type 4 set significantly less seed than plants of all other clone types except clone type 5. Only the cross involving clone type 10 as the pollen recipient and clone type 3 as the pollen donor resulted in some seed set in every cross.

No overall trend in seed set values through time was noted. No seed was set in flowers which were bagged and left unpollinated.

The results of the investigation into the swollen carpels have not been tabulated. Out of 34 emasculated flowers, none set seed and no swollen carpels were produced. Out of 34 selfed flowers, non set seed and only two produced any swollen carpels (clone type 9 produced 42 swollen carpels out of a total of 55 carpels; clone type 1 produced one swollen carpel out of a total of 31 carpels).

The unfertilized carpels in both emasculated and selfed flowers turned from green to brown with increasing age.

iii) Discussion

The numbering used to identify plant clone types in this experiment corresponds to that in Experiment 1. Only eight of the original ten clones survived to be included in this experiment. The ordering of clone types in the tables corresponds to the groups in which crosses were performed. There is variation between the numbers of crosses performed in tables because it was not always possible to carry out reciprocal crosses due to differences in flower production between plants.

The results suggest that both environmental (shown by the variation between rooms) and genetic (shown by the variation between clones) effects come into play in causing the variation seen in seed set values in Experiment 1.

Examination of the swollen carpels from Experiments 1 and 2, using a dissecting microscope, showed that the ovule inside the carpel had not increased in size compared with ovules in carpels which had not swollen. The fact that all the observed carpels were found in flowers where self or foreign pollen was present (this includes flowers from both Experiments 1 and 2) suggests that stimulation by pollen is required in their formation.

It would appear that in most flowers incompatibility mechanisms come into operation almost immediately, since in even the youngest seed heads examined, the carpels had not swollen in size. However, in those where swollen carpels were present, it was suggested (K. G. Moore, pers. comm.) that a pollen tube may have grown down towards the ovule, stimulating the carpel to swell. When fertilization of the ovule did not occur, no further growth of the carpel took place, and the ovule did not swell at all. This suggests a later-acting incompatibility mechanism in these crosses.

3) Measuring pollen dispersal variance

i) Methods

a) Measuring pollen carryover

This experiment was conducted in a greenhouse, 30 ft x 20 ft x 20 ft (approx.) at Cleppa Park field station, University College, Cardiff, Wales. A small hive of bees was established in the greenhouse a few days before the experiment was initiated. This gave the bees time to become accustomed to conditions in the greenhouse. All foliage was removed from the greenhouse prior to the start of the experiment.

Thirty plants of one clone (e.g. clone A) were arranged in their pots in the greenhouse at a distance of 5 m from the hive entrance. A spacing of 15 plants/m^2 was used. Each plant was identified by its clone type and a number between 1 and 30. All open flowers were removed from the plants. Any flowers which showed signs of opening the next day (see Fig. 3.1) were bagged. Each flower was labelled with a numbered bag. In this way a flower could be identified by its own unique code (e.g. flower number 2 from plant 1 of clone A,: A1,2).

One plant of a different clone (e.g. clone B) was placed in front of the group of clone A plants. On the day on which the experiment was to be conducted (no more than one day after bagging

the flowers) the bags were removed from clone A plants. This was done before the bees had started to forage.

Bees were allowed to forage at will on clone A plants. If a bee visited the clone B plant, however, it was then encouraged to move onto a clone A plant and forage among the clone A plants. Its sequence of visits among the clone A plants was recorded. Once a flower had been visited, it was bagged immediately. The clone B plant was covered while the sequence of visits was being watched. This ensured that no other bees visited it un-noticed and then transferred pollen to clone A plants unrecorded. Once a bee under observation had left the patch of clone A plants, the clone B plant was uncovered and the process repeated. When all the open flowers on the group of 30 plants had been visited, another set of 30 plants was brought in, and so on, until just over 100 visit sequences had been recorded (108 in all). This took five days.

All plants were transported back to Bath and left in an unheated greenhouse until seed had set (approx. six weeks). Percentage seed set was determined for each flower.

A graph was plotted of mean seed set versus flower number in a sequence of visits. This did not include data from flowers in which no seed was set at all. It was assumed that, in such flowers, no pollen had been collected in the first place.

b) Measurements of pollinator flight distances

These data were collected at Rainbow Wood Meadow on three successive days at the beginning of June, 1988. The first day was warm and sunny and there was plenty of pollinator activity. However, although the next two days were warmish, they were not sunny, and there was much less pollinator activity. This, together with the onset of the end of the flowering season in *R. bulbosus* restricted the number of observations that could be made. The chief pollinators observed foraging were *Hymenoptera* - both bumblebees and honey bees. Species could not be distinguished.

Once a bee was seen visiting a *R. bulbosus* flower, it was followed throughout its foraging run. The flowers it visited were noted and distances between successively visited flowers were recorded. A foraging run was presumed to have ended if a bee flew a distance of greater than 15 m after leaving a flower, or if it left the field in which the study was being carried out. This process was repeated until 350 flight distances had been recorded.

Visits longer than five flowers from the first flower on which the bee was noted were split into groups of six successively visited flowers. This group included a 'pick-up' flower, plus five successively visited flowers. Each flight distance was included only once in a group. Distances from 'pick-up' to the

first to fifth flowers were then calculated by adding distances between successively visited flowers. See Fig. 4.3.

This data was combined with data for pollen carryover to give an estimate of pollen dispersal variance.

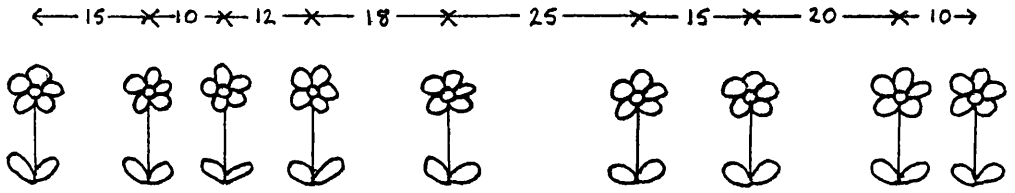
ii) Results

Mean seed set per flower decreased in a curvilinear fashion with increasing visit number in a sequence of visits by a bee after pollen pick-up from a plant of clone type B. The relationship is strong. Results are tabulated in table 4.8 and graphically presented in Figure 4.4.

Data on mean flight distances between successive flowers in a sequence of visits are summarised in table 4.9. Statistics for measuring the level of kurtosis of the distribution of flight distances were calculated using the SAS statistical package. Whilst studying bees to obtain these results, it was noted that some bees visited more than one species of *Ranunculus* and one bee also visited the flower of a plant belonging to a different family. (*Hypochaeris* sp.). These results are presented in table 4.10.

To calculate the mean ($\bar{x}$) and variance ($\sigma^2\rho$) of pollen dispersal distances, the following equations were used:

FIG. 4.3 - AN EXPLANATION OF THE CALCULATION OF BEE FLIGHT DISTANCES.



NO. OF FLOWER IN TOTAL VISIT SEQUENCE.	1*	2	3	4	5	6	7	8	9
NO. OF FLOWER IN VISIT SEQUENCE SPLIT INTO SETS OF SIX VISITS.	1*	2	3	4	5	6	1*	2	3
NO. OF FLOWER IN VISIT SEQUENCE SPLIT INTO SETS OF TWO VISITS.	1*	2	1*	2	1*	2	1*	2	1*

* IN EACH CASE 1 IS EQUIVALENT TO THE PICK-UP FLOWER.

	PICK-UP TO FLOWER NO.	DISTANCE (CM)
TOTAL VISIT SEQUENCE.	2	15
	3	25
	4	37
	5	55
	6	80
	7	95
	8	115
	9	125
SEQUENCE SPLIT INTO SETS OF SIX.	2	15
	3	25
	4	37
	5	55
	6	80
	2	20
	3	30
SEQUENCE SPLIT INTO SETS OF TWO.	2	15
	2	12
	2	25
	2	20

1) $\bar{x} = \beta_1\mu_1 + \beta_2\mu_2 + \beta_3\mu_3 + \beta_4\mu_4 + \beta_5\mu_5$

2) $\sigma^2\rho = (\beta_1\sigma_1^2 + \beta_2\sigma_2^2 + \beta_3\sigma_3^2 + \beta_4\sigma_4^2 + \beta_5\sigma_5^2) +$

$(\beta_1\mu_1^2 + \beta_2\mu_2^2 + \beta_3\mu_3^2 + \beta_4\mu_4^2 + \beta_5\mu_5^2) -$

$(\beta_1\mu_1 + \beta_2\mu_2 + \beta_3\mu_3 + \beta_4\mu_4 + \beta_5\mu_5)^2$

3) $\beta_n = \dfrac{\text{mean seed set at flower n}}{\sum \text{mean seed set at all flowers (1...5)}}$

where: β_n = probability of pollen being deposited and achieving fertilization at flower n

μ_n = mean flight distance for visit n in a sequence of visits

σ_n^2 = variance around mean flight distance for visit n in a sequence of visits (equivalent to SD^2, Crawford, 1984 a and/or b)

This method does not assume statistical independence of visits. Statistical independence should not be assumed due to possible clumping of the distribution of plants and the effects of bee behaviour.

The calculated results were:

1) $\overline{x}$ = 115.55 cm

2) $\sigma^2\rho$ = 19335.89

iii) Discussion

There are some limitations to the results which should be highlighted and discussed.

Several other groups of insects apart from bees (*Hymenoptera*) have also been observed visiting buttercup flowers, e.g. members of the orders *Lepidoptera* and *Coleoptera* (butterflies and beetles) and of the families *Syrphidae, Muscidae* and *Calliphoridae* (Hoverflies, Houseflies and Bluebottles). Bees may only account for a limited percentage of pollen flow in *R. bulbosus*. Butterflies effect longer distance pollen flow (Schmitt, 1980) perhaps flying many meters between flower visits.

TABLE 4.8

MEAN SEED SET PER FLOWER
IN A SEQUENCE OF BEE VISITS

NUMBER OF FLOWER IN SEQ. OF VISITS	MEAN SEED SET PER FLOWER (%)	STANDARD DEVIATION
1	20.79	16.36
2	9.80	8.62
3	4.24	3.66
4	2.33	2.07
5	1.98	1.49
6	0	0

N.B. This does not contain data from visit sequences where no seed was set at all.

FIG. 4.4

RELATIONSHIP BETWEEN MEAN PERCENTAGE SEED SET AND NUMBER OF FLOWERS VISITED IN A SEQUENCE SINCE POLLEN PICK-UP

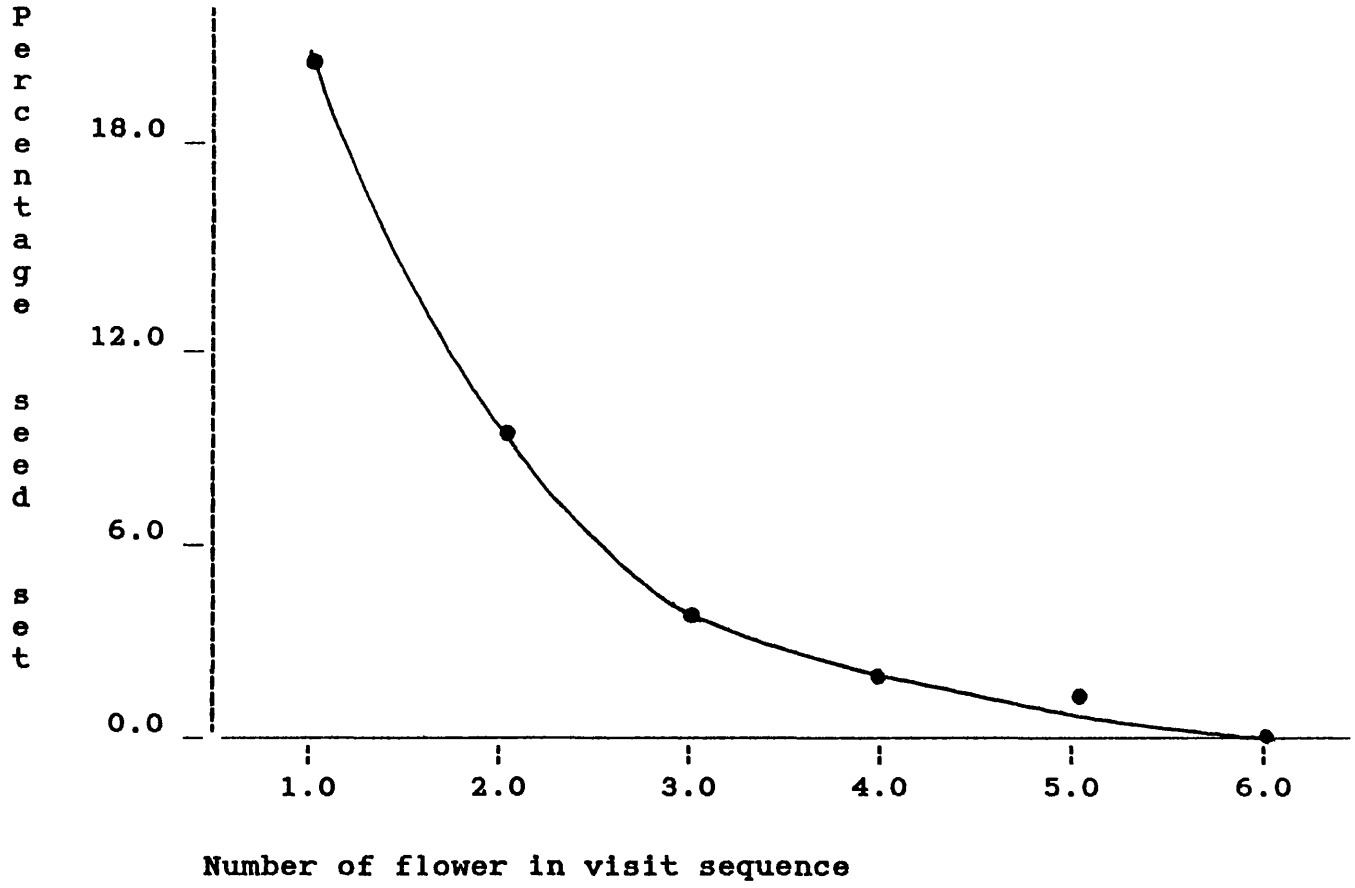


TABLE 4.9

MEAN FLIGHT DISTANCES OF BEES IN A SEQUENCE OF FIVE VISITS

VISIT SEQUENCE	NUMBER OF OBSERVATIONS	MEAN FLIGHT DISTANCE (cm)	MINIMUM FLIGHT DISTANCE (cm)	MAXIMUM FLIGHT DISTANCE (cm)	STANDARD DEVIATION	KURTOSIS
1*→ 2	308	65.65	3.00	538.00	84.13	8.17
1*→ 3	270	124.41	12.00	767.00	123.34	5.68
1*→ 4	63	184.20	26.00	682.00	154.90	2.48
1*→ 5	63	241.30	52.00	912.00	185.90	3.01
1*→ 6	46	298.30	70.00	1,220.00	226.10	5.50

* 1 represents flower from which pollen was picked up.

When all data grouped kurtosis value = 8.89

TABLE 4.10

VISIT SEQUENCES WHICH INVOLVED VISITS TO MORE THAN ONE SPECIES OF BUTTERCUP

BEE NUMBER 1		BEE NUMBER 2				BEE NUMBER 3	
NUMBER OF VISITS IN SEQ.	SPECIES VISITED	NUMBER OF VISITS IN SEQ.	SPECIES VISITED	NUMBER OF VISITS IN SEQ.	SPECIES VISITED	NUMBER OF VISITS IN SEQ.	SPECIES VISITED
1→3	*R. repens*	1	*R. acris*	74→76	*R. acris*	1→3	*R. acris*
4→6	*R. bulbosus*	2	*R. repens*	77→81	*R. bulbosus*	4→6	*R. repens*
7→9	*R. acris*	3	*R. acris*	82→84	*R. acris*	7	*R. acris*
10	*R. bulbosus*	4→6	*R. repens*	85	*R. bulbosus*	8→17	*R. repens*
11	*R. repens*	7	*R. acris*	86→87	*R. repens*	18→20	*R. acris*
12→14	*R. acris*	8→10	*R. repens*	88	*R. acris*	21	*R. bulbosus*
15→16	*R. repens*	11→13	*R. acris*	89→90	*R. repens*	22	*R. repens*
17	*R. acris*	14→40	*R. repens*	91	*R. bulbosus*	23	*R. bulbosus*
18→20	*R. bulbosus*	41	*R. acris*	92→98	*R. repens*	24→25	*R. acris*
21→24	*R. repens*	42→69	*R. repens*	99	*R. bulbosus*		
		70→71	*R. bulbosus*	100	*R. repens*		
		72→73	*Hypochaeris sp.*	101	*R. acris*		

The movements of *Diptera* (Hoverflies, Houseflies and Bluebottles) are more erratic and quicker than those of bees. Beetles often crawl, rather than fly, between flowers. These differences in behaviour will lead to different patterns of pollen flow within and between plant populations. However, they also make such pollinators more difficult to follow than bees, which can easily be observed when foraging. The use of bees provided another advantage in the availability of domesticated bees to study pollen carryover.

The conditions in which the bees were kept during the investigation into pollen carryover were very artificial. However, every attempt was made to minimize the effects. For this reason, a large greenhouse was used and the hive was orientated so that the entrance faced north-east. In order to have a population of bees more adapted to the artificial conditions of the experiment, only bees newly emerged in the greenhouse were used. However, despite the precautions, one difference in foraging behaviour was noticed between bees in the greenhouse and those outside. Fewer flowers were visited in the greenhouse per foraging bout than in the field. It is not known if foraging bouts varied in time spent, since no measurements of time were taken. The noted difference could also have been due to differences in reward value to the bees between plants in the greenhouse and those in the field (flowers in the greenhouse being less depleted than those in the field). Again, no measurement of reward value was made, so this can only be speculation.

Comparison of the results with a mean seed set value for 200 seed heads collected from Rainbow Wood Meadow (mean 51.38%; range 0 - 100%) shows that seed set values in this experiment are low. This is probably because only one bee visit was allowed per flower. In reality, flowers are probably visited several times by various insects, with pollen deposited during each visit.

The way in which flight distances between pollen pick-up and the third, fourth and fifth flowers in the visit sequence were calculated did not give absolute flight distances. Linearity of flight pattern was assumed when this may not have been so. In most cases observed, the bees did tend to approximate to this assumption. However, in any interpretation of the results it should be remembered that measurements of flight distances beyond the first flower visited after pick-up may be over-estimates.

Bees were considered lost if they moved more than 15 m between visits (it was very difficult to follow such movements). However, such flight distances could constitute long-distance pollen dispersal which was, therefore, not being measured. This should also be considered in any interpretation of the results.

Despite the limitations mentioned above, this method of measuring pollen flow has distinct advantages over simply using pollinator flight distances as a measure of pollen flow (Levin and Kerster, 1969 a). The measurements taken in this study include estimates of pollen success (estimated by percentage seed set) and

pollen carryover. Levin and Kerster (1969 a) assume that "most of the pollen acquired on one plant is deposited on the next one visited" and do not include estimates of seed set values after pollen deposition. Thomson (1986) and Thomson and Plowright (1980) show that pollen grains may be transported well beyond the first flowers visited but make no estimates of how effective such transport is in terms of seed set.

In reality a bee will be carrying pollen from more than one plant, since it will pick up and deposit pollen at each flower visited. Thus pollen deposited on a flower may be derived from several male parents. To mimic this situation accurately in an experiment to study the fates of the various pollen types would require a marker by which seeds from the various crosses could be identified. This experiment studies the fate of pollen from only one pick-up source. However, it does approximate to the situation of pollen from several sources being present and diluting the pollen picked up from the first visit. Pollen picked up from the 'female' plants whilst the bee was foraging would have diluted pollen from the 'male' plants which the bee was carrying. Since the plants do not self-fertilize, the competition occuring between these two types of pollen to fertilize ovules would not be the same as competition between pollen from two foreign sources.

Visit sequences in which no seed was set at all were not included in the analysis, because in these cases it was assumed that no pollen had been picked up in the fist place. If no pollen

had been picked up it would be inaccurate to include the results in an analysis looking at pollen deposition.

Only *R. bulbosus* plants were visited in sequences used to calculate pollinator flight distances in the field. However, it should be noted that bees do sometimes visit more than one species of plant in a visit sequence. Campbell (1985) presents evidence to show that such behaviour can reduce pollen dispersal distances and outcrossing. She shows that as the density of the 'competing' (for pollinator visits) species increases, so pollen dispersal distances and outcrossing decreases. This suggests that later in the flowering season of *R. bulbosus*, when densities of *R. acris* and *R. repens* increase, pollen flow in populations of *R. bulbosus* may decrease. This could have important consequences for plants flowering later in the season. This will be discussed further in the general discussion.

4) Measuring seed dispersal variance

i) Method

A small area of ground was prepared by removing all weeds and smoothing the soil surface until reasonably even. Ten plants which had already set seed were buried in the ground in their pots at a spacing of 2 m, such that the surface of the soil in the pot was level with the ground surface. The plants were each surrounded with a 1 m^2 piece of muslin coated in high temperature grease. The coating of grease ensured that the dispersed seeds stuck to the surface of the muslin once shed from the plant.

The plants were visited three times during a six week period until all seeds had either dispersed or smutted and rotted. The positions of the dispersed seeds were noted. This was done using tape measures stretched from stakes at the bottom two corners of the muslin squares. The tapes were crossed at the position of the seed and the measurements on the tapes at this point noted to the nearest 0.5 cm. This gave each seed a unique co-ordinate. Once the position of the seed had been noted, it was removed. The position of the centre of each parent plant was calculated in a similar way. The co-ordinates determined from the tapes were then converted into x and y co-ordinates using a computer program (N. Watson, pers. comm.). From this it was possible to measure the parent-seed dispersal distances by using the simple equation

$$d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$$

where: d = distance between two points with co-ordinates (x_1, y_1), (x_2, y_2)

point (x_1, y_1) = plant co-ordinate

point (x_2, y_2) = seed co-ordinate.

ii) Results

The overall mean seed dispersal distance for the ten plants was 27.54 cm. The pattern of dispersal for each plant is plotted in Figures 4.5 to 4.14.

A one-way ANOVA (Genstat, version 5) was used to investigate possible differences between plants in the distances over which they disperse their seeds. A significant difference ($p < 0.001$) between plants was found.

Statistics for measuring the level of kurtosis of the distribution of seed dispersal distances were calculated for each plant and overall.

Seed dispersal in relation to number of plant diameters moved by the seed was calculated following calculations done by Levin (1981). Mean plant diameter was calculated from values recorded for experiment c) in Chapter 3. The value was 9.61 cm (SD = 3.22). The results are recorded in table 4.11.

iii) Discussion

The results suggest the occurence of very localized seed dispersal in terms of absolute distances moved by the seeds from the parent plant. This conclusion is mirrored when results are discussed in terms of number of plant diameters moved by a seed. Seeds disperse, on average, a distance equivalent to between only 2 and 3 plant diameters. This is lower than values found for *Phlox pilosa* (4 plant diameters), *Liatris aspera* (12 plant diameters), and *Eucalyptus regnans* (5 plant diameters) (Levin, 1981).

It was realised that seeds may be dispersed further than the area covered by the muslin. For this reason, the soil around the muslin squares was examined for the presence of *R. bulbosus* seeds. None were found in the immediate vicinity.

There are other factors operating under field conditions which may effect longer-distance dispersal. For instance, dispersal by animal vectors. It was noted at the field site that

PLANT NUMBER	NUMBER OF SEEDS DISPERSED	SEED DISPERSAL DISTANCE (cm)					DISPERSAL IN NUMBER OF PLANT DIAMETERS
		MEAN	STANDARD DEVIATION	MIN.	MAX.	KURTOSIS	
1	205	22.80	5.07	1.02	43.29	3.84	2.37
2	115	27.55	9.29	7.01	48.17	0.05	2.87
3	197	28.58	4.07	18.91	48.13	7.15	2.97
4	60	19.50	6.59	7.93	30.88	-0.83	2.03
5	130	27.83	10.37	2.04	48.23	-0.62	2.90
6	43	29.32	10.04	11.02	42.55	-1.38	3.05
7	106	31.72	6.31	8.58	47.99	1.19	3.30
8	160	31.51	9.71	7.38	58.19	-0.01	3.28
9	78	21.81	6.19	4.14	34.44	-0.28	2.27
10	111	32.09	12.19	7.44	52.15	-0.70	3.34
OVERALL	1205	27.54	8.91	1.02	58.19	0.50	2.86

TABLE 4.11

SUMMARY OF SEED DISPERSAL FOR TEN EXPERIMENTAL PLANTS

Analysis of variance on dispersal distances by plant gave an F value of 27.42 ($p < 0.001$).

Figs. 4.5-4.14 Seed Dispersal Patterns For Ten Experimental Plants.

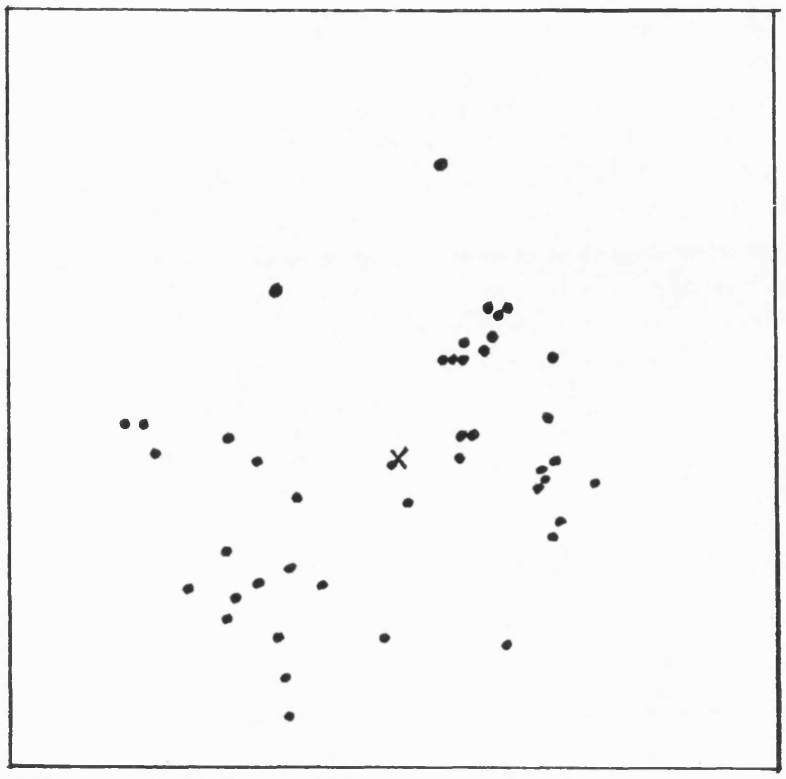


Fig. 4.5 Plant 1

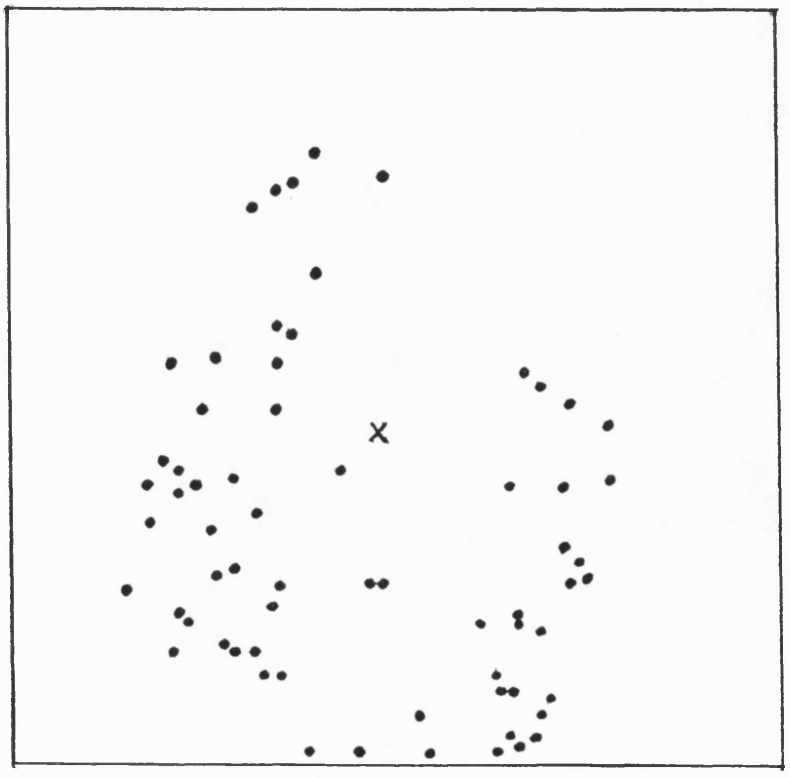


Fig. 4.6 Plant 2

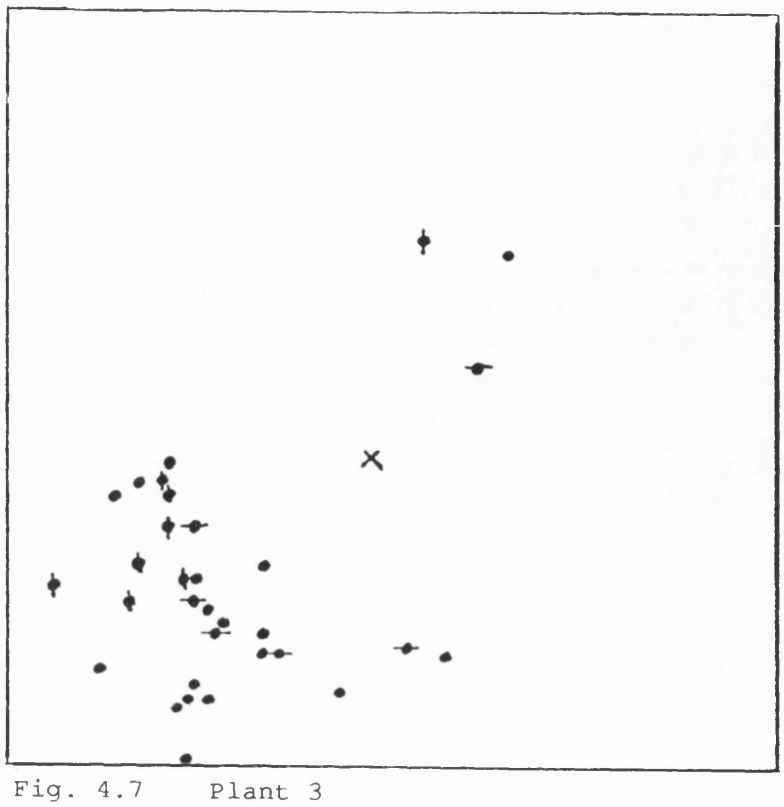


Fig. 4.7 Plant 3

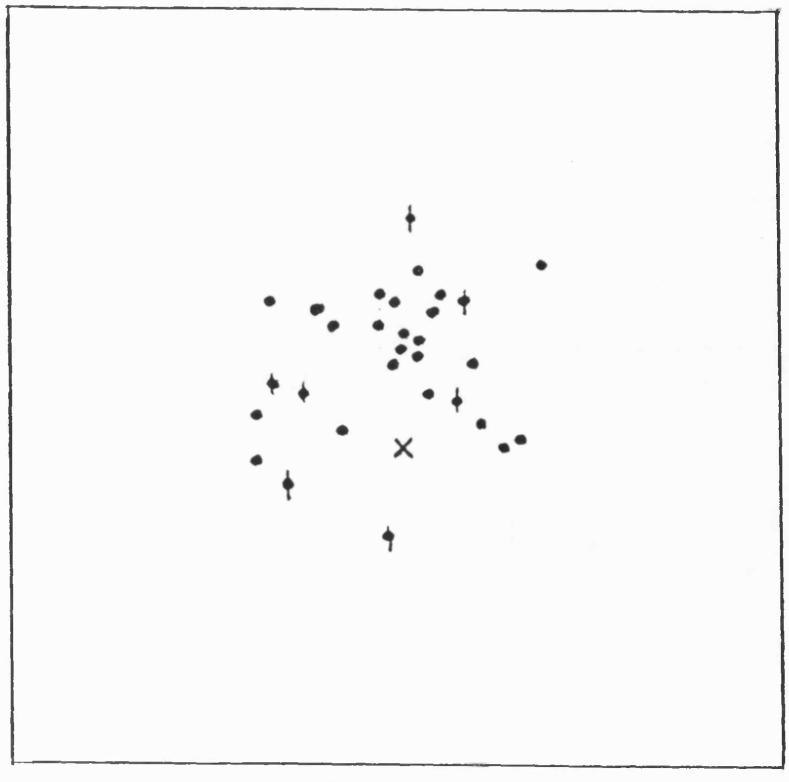


Fig. 4.8 Plant 4

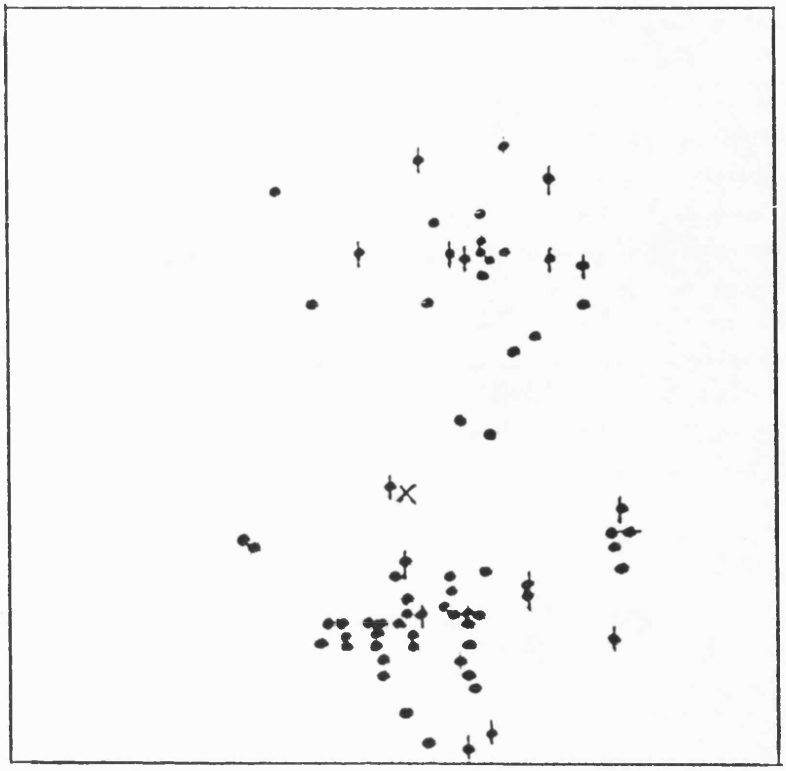


Fig. 4.9 Plant 5

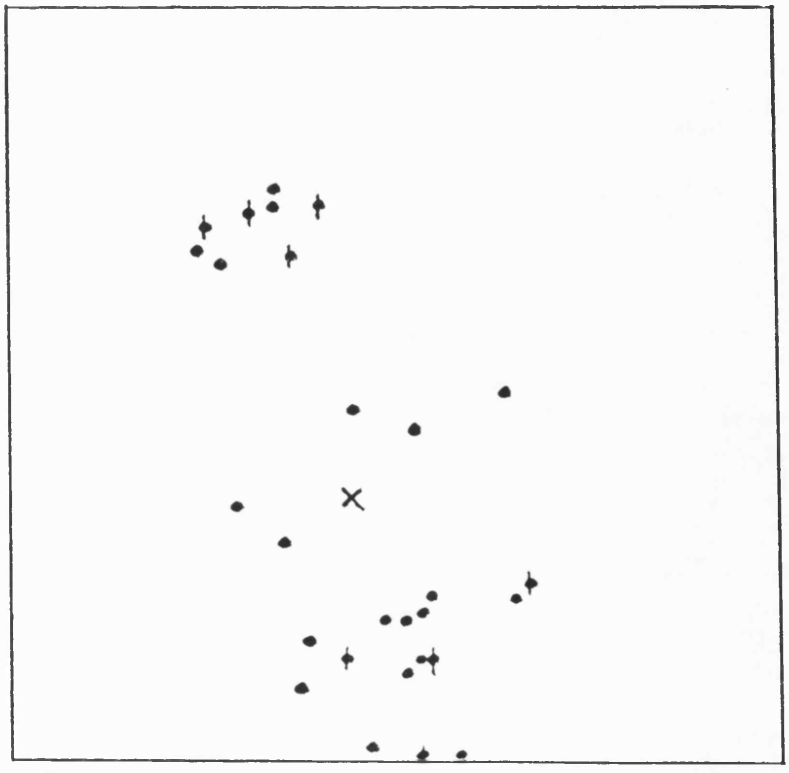


Fig. 4.10 Plant 6

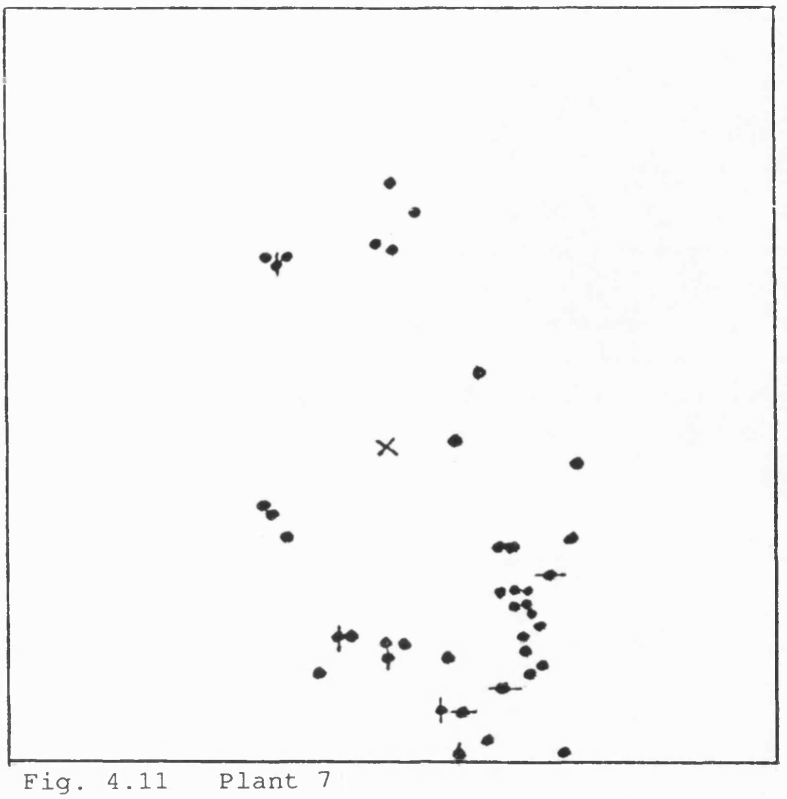


Fig. 4.11 Plant 7

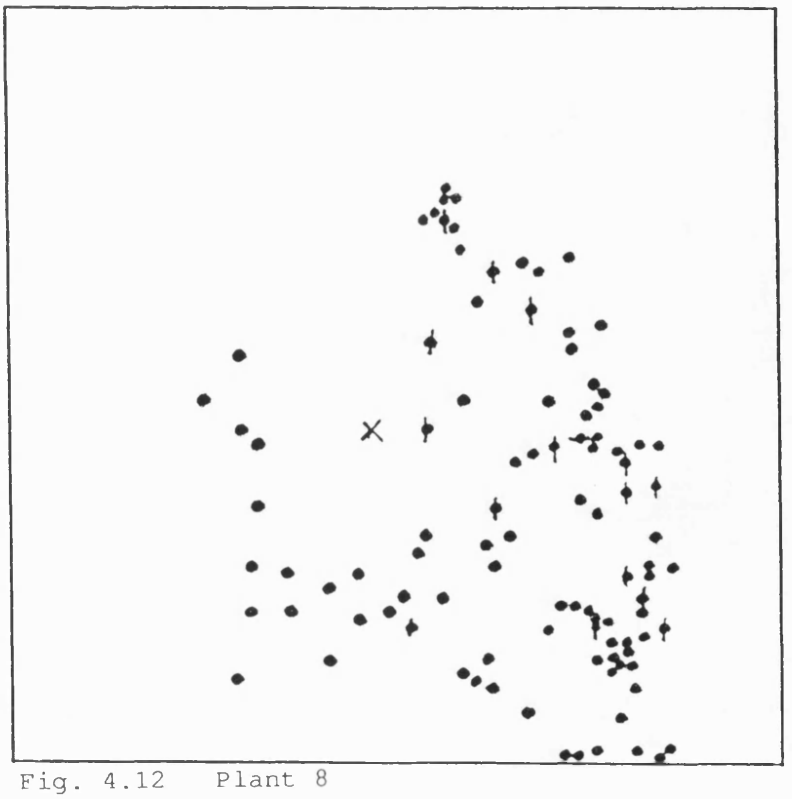


Fig. 4.12 Plant 8

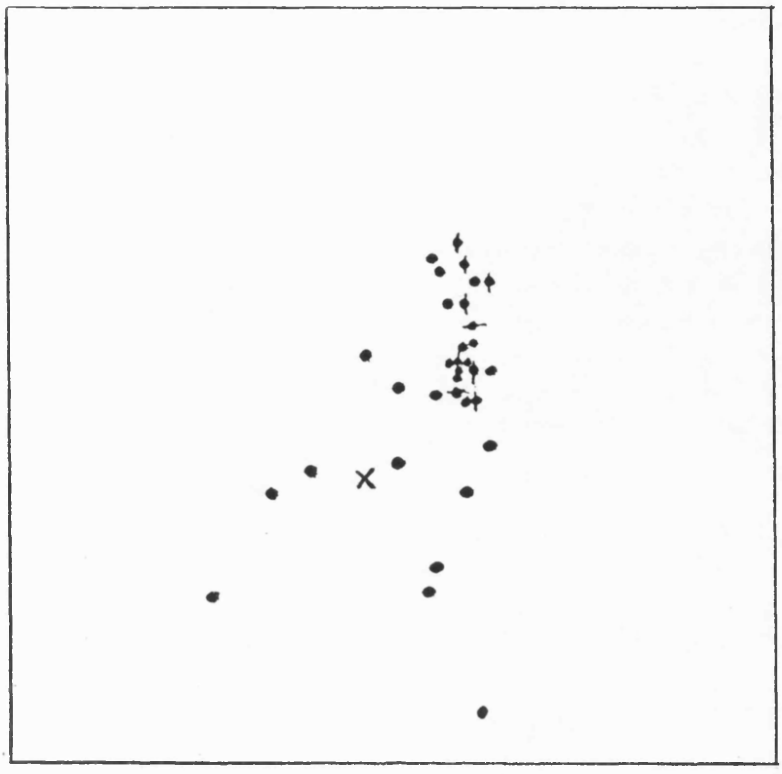


Fig. 4.13 Plant 9

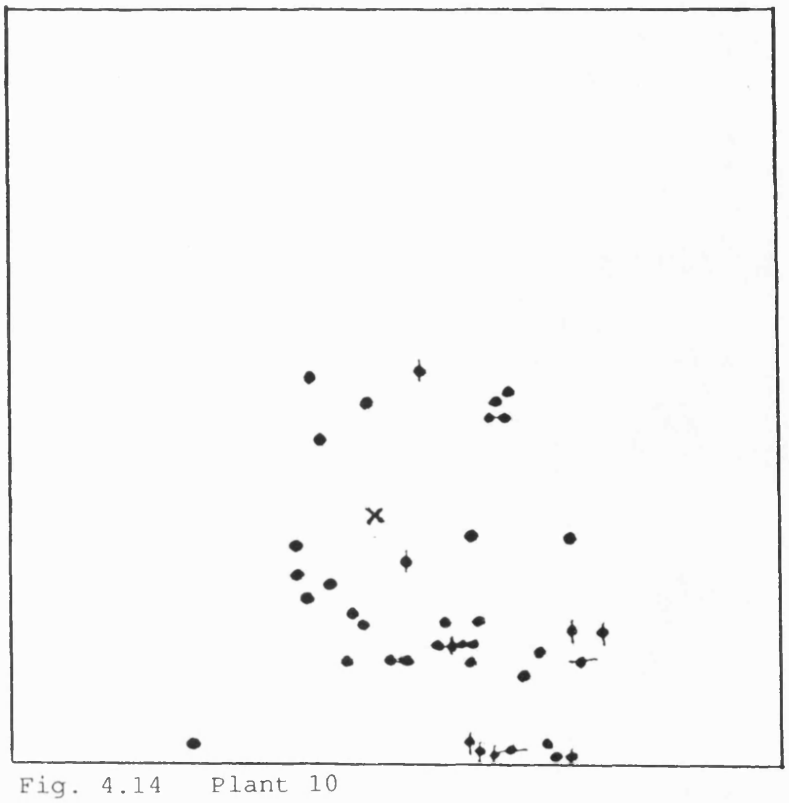


Fig. 4.14 Plant 10

some seed heads were being eaten by rabbits, even though *R. bulbosus* contains high levels of ranunculin. If seeds were able to pass through the guts of the rabbits intact, movement away from the parent plant and consequent defaecation by the rabbit could promote long-distance seed dispersal. To investigate this further, 200 rabbit droppings were collected and examined for the presence of intact *R. bulbosus* seeds. Although many pieces of seed were found, no intact seeds were recovered. This suggests that seed dispersal by this method does not occur.

Animal vectors may promote seed dispersal in other ways. Seeds may become lodged in the fur of animals or in mud on their feet. On several occasions after coming back from the field site, *R. bulbosus* seeds were found trapped in clothing and hair. Animals may also knock seeds heads as they walk past them, projecting the seeds into the air and thus causing longer-distance dispersal.

Dispersal around the experimental plants was not very uniform (see Figs. 4.5 to 4.14). This is probably due to dispersal occuring when the base of the flowering stem rots, causing it to fall over, depositing seeds where it landed. There were significant differences between plants in their mean dispersal distances. Some of this variation may have been due to differences between plants in the heights of flowering stems. Taller plants will disperse seeds further compared with shorter plants (Levin and Kerster, 1974) especially if the majority of

seed dispersal is achieved by the method just described. The height of the flowering stems was not measured, so this relationship cannot be investigated. Simple observation of *R. bulbosus* plants reveals apparent differences between plants in flowering stem height. Seed dispersal by stem rotting resulted in many of the seeds falling in groups of between 2 - 35 seeds. This was also observed in the field. Competition between seedlings germinating from such groups of seeds would be intense. It is probable that only one seedling from such a group would survive to maturity. However, the theory of kin selection may hold some clues as to why this should occur.

The theory of kin selection was developed using animal systems as models (Hamilton, 1964). Since then, the theory has been expounded by a number of authors (Hepper, 1986; Michod, 1982; Nakamura, 1980; Taylor, 1988). Michod (1982) reviews several theoretical studies on kin selection and identifies a pre-requisite for the occurence of evolution of a genetic trait by kin selection. That is, "to evolve by kin selection, a genetic trait expressed by one individual (the actor) must affect the genotypic fitness of one or more individuals who are genetically related to the actor in a non-random way at the loci determining the trait".A later paper by Hepper (1986) supports this pre-requisite but adds that kin recognition must also occur. He goes on to discuss the possible mechanisms of kin recognition in animals. Animals have an apparent advantage over plants in the ability to recognise kin.

They often possess senses such as sight, smell, touch and hearing, all of which could be used in kin recognition.

The mechanisms of kin recognition in plants are more difficult to explain. A plant may have to rely more on the probability of relatedness to particular individuals. For instance, where gene flow is restricted, the probability of close neighbours being related is great. A plant displaying behaviour which favours its neighbour over itself is, therefore, more likely to be helping a relative than if it displayed the same behaviour towards a conspecific which was growing much further away. If, in helping its relative, it increases its inclusive fitness, the trait for 'altruistic' behaviour towards its neighbour will be selected for. Of course, there will be times when the plant may be growing next to an unrelated individual. If this occurs very often, the trait for apparently 'altruistic' behaviour will experience a negative selection pressure and eventually be lost from the gene pool.

Nakamura (1980) reviews the impact of kin selection on plant evolution. He shows that it may operate to restrain competition, promote altruism, and regulate parent-offspring behaviour.

In using the kin selection model to explain why seeds should be dispersed in groups, it must first be ensured that the criteria for the occurence of kin selection are met. The seeds are related through the maternal parent and possibly through the paternal

parent. The latter is more likely if seeds are derived from the same seed head. The 'trait' (Michod, 1982) which is being examined is seed dispersal in clumps.

There may be an advantage to the seeds in dispersing in clumps. For instance, a certain amount of pressure might be required for seedlings to be able to break through the soil surface. One seedling, on its own, may not be able to exert this pressure, whereas a group of seedlings together could. Seeds being dispersed singly may, then, not be able to germinate, whereas at least one of those in a group will. In this case, any individual in the group which does not survive to maturity can only gain inclusive fitness through its surviving sibs. However, this inclusive fitness value will be greater than it would otherwise have been if seeds had dispersed singly and none had germinated.

Taylor (1988) discusses seed dispersal in these terms. In his model, there is a disadvantage to dispersal (e.g. low availability of suitable sites for seedling establishment) which is outweighed by the advantage to the related individuals left behind conferred by the dispersing individual (e.g. reduced competition).

There may be conflicting interests between the mother and her offspring in dispersal patterns. The theory of parent-offspring conflict is discussed by Ellner (1986), with reference

to the timing of germination, and by Mazer (1987), Temme (1986) and Westoby and Rice (1981), with reference to the garnering of resources by seeds during their development.

In the case of seed dispersal, it may be that it is in the interests of the seeds to disperse in groups (as discussed above). However, it may be in the mothers' interests to disperse seeds singly so as to, for example, decrease competition between sibs.

A simple experiment was performed to investigate if there is any advantage conferred on the seeds, in terms of probability of germination, when they disperse in clumps. Seeds were planted in a grid pattern in trays of Fisons' M2 compost. They were planted in groups of 1, 2, 4, 8, 16 and 32, at a depth of 1.6 cm, with a spacing of 3.5 cm between groups of seeds. A total of 32 seeds were planted for each group size, i.e. 32 single seeds, 16 groups of 2, 8 groups of 4, 4 groups of 8, 2 groups of 16, and one group of 32. Four replicates of this pattern were used. Within groups, seeds from the same seed head were used. The number of germinated seeds in each groups was recorded every 2 - 3 weeks until eleven weeks after starting the experiment. The results are presented in table 4.12.

The sample sizes were very small and the experimental design not robust - seeds were not randomized within the trays. A Kruskal-Wallis non-parametric test was performed on the data to assess any overall differences between mean number of germinated

TABLE 4.12

SEED GERMINATION RATE WHEN SEEDS ARE PLANTED SINGLY OR IN GROUPS OF VARYING NUMBER

	NUMBER OF SEEDS IN SEED GROUP					
	1	2	4	8	16	32
TOTAL NUMBER GERMINATED TRAY 1	28	28	26	27	21	21
TOTAL NUMBER GERMINATED TRAY 2	28	30	25	23	25	0
TOTAL NUMBER GERMINATED TRAY 3	19	25	27	24	28	25
TOTAL NUMBER GERMINATED TRAY 4	18	21	15	16	17	18
TOTAL NUMBER GERMINATED OVERALL	93	114	93	90	91	64
MEAN GERMINATION OVER ALL TRAYS	23.2	26.0	23.2	22.5	22.7	16.0
SD	5.5	3.9	5.6	4.6	4.8	11.0

A Kruskal-Wallis test on the means gave H = 4.132. That is, the means were not significantly different from one another ($p > 0.05$).

seeds in the different sized groups. No significant difference was found ($p > 0.05$). So, it would appear that planting the seeds in groups of more than one seed did not confer any advantage on the seeds in such groups in terms of probability of germination. This suggests that it is in the mothers', rather than the offsprings', interests to have the seeds disperse in groups. However, further investigation of this area is warranted before conclusions can be drawn. Survival rate of the seedlings was not investigated at all. Once the seedlings had reached a particular size, it became impossible to carry on with the experiment, since the groups of seedlings started to overlap with each other. Investigation of survival rate may have revealed a positive disadvantage to group dispersal, as competition between seedlings for resources came into play. Conditions for germination were ideal. Under poorer conditions (e.g. compacted soil) an advantage to group dispersal may have been highlighted.

5. Measuring vegetative density

i) Method

The vegetative density of *R. bulbosus* was measured in eight separate 30 m^2 plots chosen at random within the Rainbow Wood Meadow population. Four plots were chosen in each of two successive years. Random points were selected within each of these plots by generating random pairs of co-ordinates on a computer. A 25 cm^2 quadrat was placed with the bottom left-hand corner on the selected point and the number of plants within that area was counted. A quadrat of size 25 cm^2 was chosen because it was not certain that the distribution of plants was random. When in doubt about the nature of the distribution, Greig-Smith (1983) advises the use of the smallest quadrat practicable. The quadrat was made of flexible rubber tubing with corners of fixed 90° angle. This permitted the quadrat to fit around the undulating surface of the field without changing its area (as long as the 90° angles remained constant). The density at a minimum of 60 random points was measured in each plot. The final number of points used was determined by calculating the running mean. Once there was less than 3% variation in a series of 4 means, the mean at that point was considered to be an accurate estimate of the true mean.

ii) Results

The results for both years are summarised in table 4.13. For both years, there was variation in vegetative density between the four plots used. The quadrats which fell on anthills and footpaths were marked and mean densities for these calculated separately, as well as being included in the overall calculation of vegetative density.

A one-way analysis of variance, using Genstat5 was used to perform tests of significance on differences between mean vegetative densities for the two years (1986 and 1987). The density recorded in 1986 (88.64/m^2) was found to be significantly higher than that recorded in 1987 (59.36/m^2) ($F = 45.59$, $p < 0.001$).

Similar tests were used to investigate differences between mean vegetative densities on anthills and footpaths compared with overall vegetative density. Vegetative density on footpaths was only measured in 1987, and was 114.40/m^2, which is significantly higher ($F = 22.85$, $p < 0.001$) than that for overall vegetative density for 1987 (59.36/m^2). Vegetative density on anthills was measured in both 1986 and 1987. The data from the two years were pooled to increase sample size. No significant difference was found between vegetative density on anthills (69.28/m^2) and overall vegetative density (74.72/m^2) ($F = 0.23$, $p = 0.635$).

TABLE 4.13

VEGETATIVE DENSITY OF *R. BULBOSUS L.* AT

RAINBOW WOOD MEADOW IN 1986 AND 1987

DATE/PLOT	Number of quadrats used	Mean plants per 25 cm^2	Mean plants per m^2	S.D.
1986/A	90	6.12	97.92	3.49
1986/B	60	6.92	110.72	3.71
1986/C	70	5.81	92.96	4.09
1986/D	71	3.37	53.92	3.37
1986/ anthills	15	4.73	75.68	3.71
1986/ overall	291	5.54	88.64	3.62
1987/1	70	4.59	73.44	1.79
1987/2	70	2.66	42.56	1.40
1987/3	70	3.90	62.40	1.79
1987/4	70	3.71	59.36	1.46
1987/ anthills	9	3.67	58.72	3.32
1987/ footpath	13	7.15	114.40	1.87
1987/ overall	280	3.71	59.36	2.76
Both years overall	571	4.64	74.24	3.35

iii) Discussion

Although vegetative density is not used in the neighbourhood calculations, it was thought that it would be of interest to measure it. The area in which the study was conducted (Rainbow Wood Meadow) is interesting for the large number of anthills which abound throughout the field. In some species (e.g. *Primula vulgaris*, Cahalan, 1983) ants are involved in seed dispersal, effecting longer distance dispersal than the plants could manage by themselves. It was thought that *R. bulbosus* seeds could be similarly dispersed. This is why quadrats which fell on anthills were marked. Although the sample sizes were very different, the results collected here suggest that ants are not involved in dispersal of *R. bulbosus* seeds to any great extent.

Another feature of the field is that it has a footpath running through it. Harper (1957) stated that where footpaths cross fields "buttercup free lanes conspicuous in the flowering season" are visible. For this reason, in the second year of study (1987) any quadrats which fell on a footpath were noted, and the mean vegetative density within these compared with the mean overall density. Again, sample sizes were very different. However, a highly significant difference was revealed, with the density of *R. bulbosus* on footpaths being higher than overall density. This contradicts Harper's conclusion. However, it may be that only flowering density is reduced on footpaths, not

vegetative density. This will be discussed further in the next section.

6) Measuring flowering density and sequence

i) Methods

The method used to measure flowering density was the same as that used to measure vegetative density, except that the number of flowering plants in each quadrat, rather than the total number of plants, was counted. The number of flowers on each flowering plant was also counted. Flowering density was measured for four plots in only one year (1988).

The sequence of flowering was measured in a 'permanent' quadrat in one area of Rainbow Wood Meadow. The top right-hand corner of a 10 m^2 area was marked using a large wooden stake driven into the ground. This ensured that the position of the plot could be found on subsequent visits. Ten points were marked within the larger plot. The position of these points was determined using pairs of random co-ordinates. A 0.5 m^2 quadrat was laid down at each of the ten points. The bottom left corner of the quadrat was placed on the selected point. The positions of all *R. bulbosus* plants within this area were noted using a system of co-ordinates. The co-ordinates were determined using a scale marked on the sides of four smaller quadrats within the 0.5 m^2 quadrat. Each plant thus had a unique co-ordinate and could, therefore, be identified on subsequent visits. See Fig. 4.15.

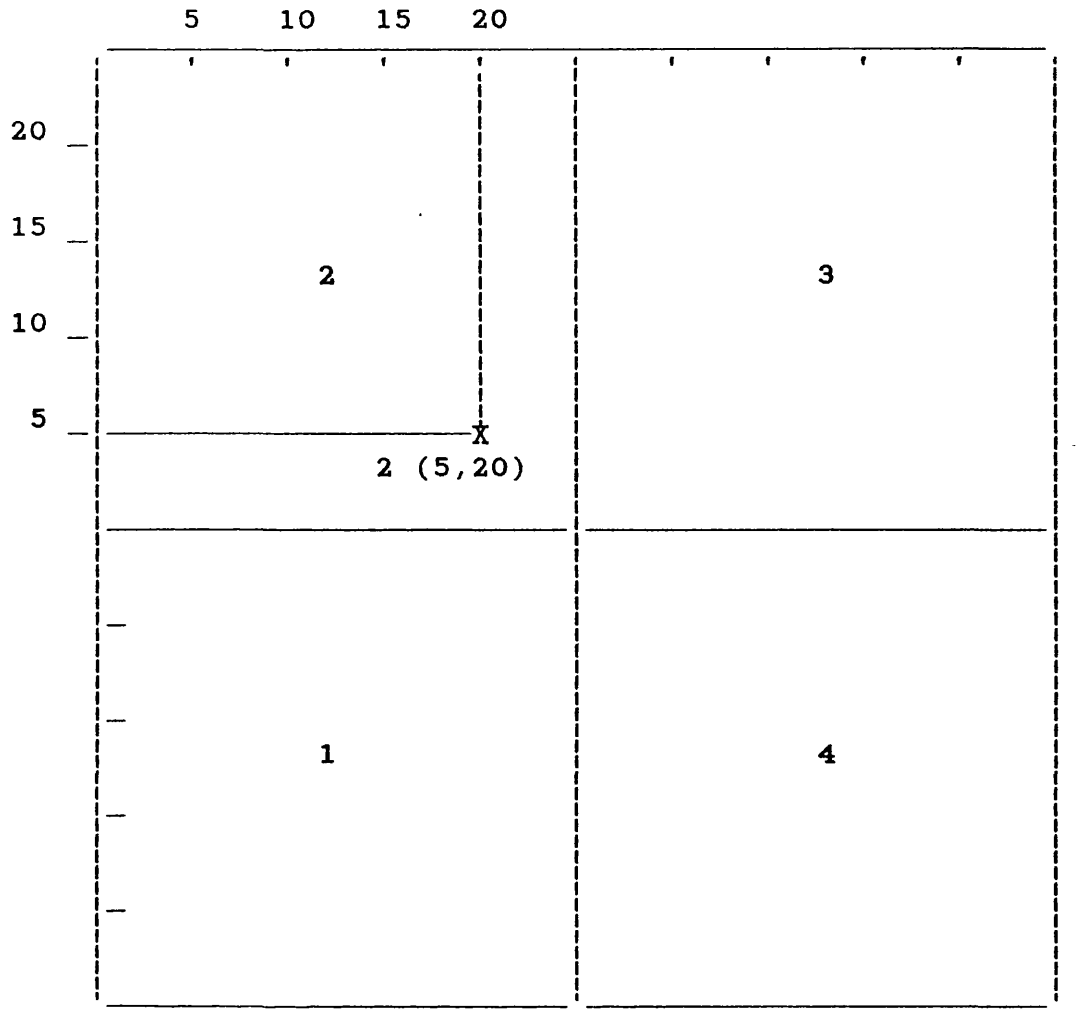


FIG. 4.15 Diagram to show the determination of plant co-ordinates

The site was visited at weekly intervals during flowering. On each visit it was noted whether or not the plant was flowering and, if so, how many flowers it possessed.

ii) Results

Flowering density was measured on two days, 6/5/89 and 17/5/89. On the first day, four plots were used. On the second day, only three. Although the plots on the two dates were in

similar areas, absolute consistency could not be achieved. As with vegetative density, flowering densities in quadrats falling on footpaths or anthills were recorded separately as well as being included in the overall measurements of density. The results are presented in table 4.14. Genstat5 was used to perform significance tests on the differences between the mean flowering densities on the two days on which recordings were made. The flowering density on 17/5/89 (27.2/m^2) was found to be significantly higher ($F = 75.95$, $p < 0.001$) than the flowering density recorded on 6/5/89 (17.92/m^2).

Genstat5 was also used to perform significance tests on differences between mean flowering density on anthills and footpaths compared with overall mean flowering densities. In both these cases results from the two dates were combined to increase sample size. Mean flowering density on anthills (7.04/m^2) was found to be significantly lower ($F = 7.24$, $p = 0.007$) than mean flowering density overall (17.92/m^2). No significant difference ($F = 1.46$, $p = 0.228$) was found between mean flowering density on the footpath (12.32/m^2) and overall flowering density (17.92/m^2).

Flowering density in the 10 m^2 permanent plot varied greatly between quadrats within the plot. For instance, in quadrat 1, 88.9% of plants produced flowers, whereas only 21.05% produced flowers in quadrat 3. Overall, mean flowering density across quadrats and dates was 36.5/m^2 ($n = 10$, $SD = 5.99$). These data are not included in the overall measure of flowering density. The

TABLE 4.14

FLOWERING DENSITY OF *R. BULBOSUS L.* AT RAINBOW WOOD MEADOW ON TWO DAYS IN 1988

Date/Plot	Number of quadrats used	Mean flowering plants/25 cm^2	Mean number flowers/25 cm^2	Mean flowering plants/m^2	Mean number flowers/m^2
6/5/88 1	70	0.90	1.43	14.40	22.88
6/5/88 2	70	0.71	0.86	11.36	13.76
6/5/88 3	70	0.43	0.50	6.88	8.00
6/5/88 4	70	0.69	0.77	11.04	12.32
6/5/88 overall	280	0.67	1.07	10.72	27.20
17/5/88 1	70	1.37	2.81	21.92	44.96
17/5/88 2	70	1.96	2.91	31.36	46.56
17/5/88 3	70	1.77	3.03	28.32	48.48
17/5/88 overall	110	1.70	2.92	27.20	46.72
Both dates overall	490	1.12	1.75	17.92	28.00
Both dates anthills	25	0.44	0.84	7.04	13.44
Both dates footpath	22	0.77	0.95	12.32	15.20

majority of plants flowered between 12/5/87 and 26/5/87 with peak flowering recorded on 19/5/87. Results are summarised in table 4.15 and listed in Appendix 2.

iii) Discussion

Flowering density was lower on 6/5/88 than on 17/5/88 because the flowering season was just starting on 6/5/88. The neighbourhood size/area will be calculated using data from 6/5/88, 17/5/88 and the overall result across the two dates. This should show up some differences in neighbourhood size/area through time.

The "buttercup free lanes" which Harper (1957) referred to were visible during the flowering season where the footpath crossed the field. This is reflected in the slightly lower (not significantly) flowering density recorded in quadrats which fell on the footpath. In the context of the results obtained for vegetative density, it would appear that a higher proportion of the plants growing on the footpath failed to flower. If a similar proportion of plants on the footpath flowered as flowered overall, a higher flowering density would be expected on the footpath compared with overall. This reduction in the probability of flowering by plants growing on the footpath is probably caused by trampling. *R. bulbosus*, in adopting a low, rosette-type growth habit in its vegetative stage, may be able to survive the trampling. However, other species of plant (especially taller growing species) may be unable to survive. This will result in

TABLE 4.15

SUMMARY OF FLOWERING SEQUENCE
IN TEN 0.5 m^2 QUADRATS AT RAINBOW WOOD MEADOW

VISIT DATE	NUMBER OF PLANTS FLOWERING	AVERAGE FLOWERS PER PLANT (USING ONLY FLOWERING PLANTS)	% PLANTS FLOWERING
28/4/87	2	1 SD 0	1.12%
12/5/87	71	1.00 SD 0	39.66%
19/5/87	82	2.60 SD 2.54	46.07%
26/5/87	17	2.00 SD 2.29	9.55%
9/6/87	3	1.67 SD 1.15	0.02%

(Started with 179 plants in total, one of which died)

reduced interspecific competition for *R. bulbosus* plants growing on footpaths compared with those growing in surrounding areas. However, when the flowering stem is produced it is usually projected up into the air. Such a structure would be less likely to survive trampling.

Flowering density on anthills was significantly lower than that recorded overall. A possible explanation for this is that the soil on the anthills is much more disturbed, thus preventing establishment of plants or their survival to maturity and flowering. The plants which were recorded in vegetative density measurements may have been new seedlings which did not survive to reproductive maturity.

Flowering density calculated for the permanent quadrat was higher than that calculated over the other plots, as it was calculated over a period of time for the permanent quadrat but at only a single point in time for the others. That is, the number of plants flowering on day 1 plus number of new plants flowering on days 2, 3, 4 and 5, were all included in the measurement for the permanent quadrat, whereas only the number of plants flowering on one day were used in all other calculations.

The fact that most plants tend to flower synchronously has consequences regarding gene flow in the population. If all plants flower synchronously, gene flow is more likely to be restricted, since pollinators will tend to visit nearest neighbours

(Ellstrand, Torres and Levin, 1978; Levin and Kerster, 1969; Schaal, 1978; Schmitt, 1980; Zimmerman, 1982). If a few plants produce flowers earlier or later in the season and these plants are dispersed throughout the population, pollinators foraging specifically on *R. bulbosus* will be forced to travel further between flower visits. This will result in longer distance pollen flow. However, as has been discussed in a previous section, when *R. bulbosus* occurs at lower densities, bees have been shown to visit flowers of other species in between visiting *R. bulbosus* flowers, possibly resulting in lower rates of pollen deposition on subsequent *R. bulbosus* flowers. However, longer distance gene flow may still be effected.

R. bulbosus is the first of the three most common species of buttercup in Britain (*R. bulbosus, R. repens* and *R. acris*) to flower, but late flowering *R. bulbosus* plants overlap with early flowering *R. repens* and *R. acris*. All three species occur together at Rainbow Wood Meadow. Bees have been seen to visit all three species in one foraging bout (pers. obs.). Thus hybridisation is possible but not recorded, indicating that cross-pollination is not successful.

It was intended to continue the flowering sequence experiment in the following year using the same plants. However, this proved to be impossible because of movement of the stake marking the permanent quadrat. This meant that plants could not be identified from one year to the next.

4.3 CALCULATION OF NEIGHBOURHOOD SIZE AND AREA

i) Methods

The measurements of pollen and seed dispersal variances, outcrossing rate and density of reproductive individuals, were substituted into Crawford's (1984) formulae for calculation of neighbourhood size and area. Calculations were performed both with and without corrections for leptokurtosis using:

a) pollen dispersal variance allowing for pollen carryover;

b) pollen dispersal variance not allowing for pollen carryover (i.e. for the first visit only with no inclusion of seed set values);

c) flowering density measurements from 6/5/88 only;

d) flowering density measurements from 17/5/88 only;

e) overall flowering density using data collected on both dates.

The equations for calculating neighbourhood size and area without correcting for leptokurtosis are:

$$A = 4\pi \left(\frac{t\sigma_p^2}{2} + \sigma_s^2\right)$$

and

$$Ne = 4\pi \left(\frac{t\sigma_p^2}{2} + \sigma_s^2\right) \frac{d}{2} (1+t)$$

where:

A = neighbourhood area

Ne = neighbourhood size

t = outcrossing rate

σ_p^2 = variance of pollen dispersal distances

σ_s^2 = variance of seed dispersal distances

d = genetically effective density (number of flowering plants per unit area).

In the case of *R. bulbosus*, t cancels out of these equations since self-infertility has been demonstrated, so $t = 1$.

The equations for calculating neighbourhood size and area with corrections for leptokurtosis are:

$$A_1 = \left(\frac{Kp\sigma_p^2}{2} + Ks\sigma_s^2\right)$$

and

$$Ne_1 = \pi\left(\frac{Kp\sigma_p^2}{2} + Ks\sigma_s^2\right) \frac{d}{2} (1+t)$$

assuming self-infertility.

The symbols Kp and Ks represent constants which correct for leptokurtosis in pollen and seed distributions respectively. The steps involved in calculating these constants are given in Appendix 3.

ii) Results

The results of the calculations of neighbourhood size and area are presented in tables 4.16 and 4.17.

The overall seed dispersal distribution was found to be only very slightly leptokurtotic (γ = 0.5). The value of α (see Appendix 3) was -0.258. Beattie and Culver (1979) suggest that where $\alpha \leq 0.5$ the corrected equation for calculating Ne reduces to Wright's (1946) original equation and that values of $\alpha > 0.5$ indicate leptokurtosis. This value of α is much smaller than 0.5.

TABLE 4.16

VALUES OF NEIGHBOURHOOD AREA AND SIZE CALCULATED
WITHOUT ALLOWING FOR LEPTOKURTOTIC DATA

POLLEN CARRY-OVER	FLOWERING DENSITY MEASURED ON					
	6/5/88		17/5/88		2 DATES COMBINED	
	A(m^2)	Ne	A(m^2)	Ne	A(m^2)	Ne
With	12.249	131.308	12.249	333.169	12.249	219.500
Without	4.547	48.742	4.547	123.675	4.547	81.480

TABLE 4.17

VALUES OF NEIGHBOURHOOD AREA AND SIZE CALCULATED
ALLOWING FOR LEPTOKURTOTIC DATA

POLLEN CARRY-OVER	FLOWERING DENSITY MEASURED ON					
	6/5/88		17/5/88		2 DATES COMBINED	
	A_1(m^2)	Ne_1	A_1(m^2)	Ne_1	A_1(m^2)	Ne_1
With	11.207	120.136	11.207	304.823	11.207	200.825
Without	4.156	44.557	4.156	113.056	4.156	74.484

4.4 GENERAL DISCUSSION OF THE CHAPTER

Wright (1946) concluded that a value of Ne 20 will result in considerable genetic drift within sub-populations, due to both random changes in gene frequency, which become more important in small populations, and to the effects of inbreeding due to the limited pool of individuals from which mates can be drawn in small populations. Genetic drift can result in the fixation of some alleles (either deleterious or advantageous) in, and the loss of others from, the sub-populations. This reduction in genetic variability within sub-populations will reduce their ability to respond to changing environmental pressures. All values of Ne for *R. bulbosus* were greater than 20 individuals. However, many were close to, or considerably less than, 200. Wright (1946) predicts that a value of Ne 200 could possibly result in genetic drift within a sub-population. The effects would be less marked than with a value of Ne 20. Thus, it is likely that the population of *R. bulbosus* at Rainbow Wood Meadow is suffering from the effects of genetic drift due to small population size.

The estimates of effective population size obtained varied depending upon whether pollen carryover, leptokurtosis and variation in flowering densities between sample dates were included in the calculations. Taking into account pollen carryover increased the value of Ne compared with when pollen carryover was ignored (120.1 - 333.2 and 44.6 - 123.7 respectively). This difference markedly influences the

conclusions drawn about the importance of genetic drift in the population. Where possible, estimates of pollen carryover should be included in measurements of pollen dispersal distance. Simply using measurements of plant spacing (Levin and Kerster, 1969 b) to estimate pollen dispersal distances gives severe under-estimates of the level of gene flow.

Wright's (1946) formulae for calculating Ne and A assume normal distributions for gene dispersal distances. This was found not to be the case for pollen dispersal distances. Seed dispersal distances were normally distributed. Bateman (1950) discusses this problem in some detail and provides evidence from many studies to show that leptokurtic distributions are common. Taking leptokurtosis into account resulted in a slight reduction in the estimated values of Ne and A for *R. bulbosus*. This effect was also noted by Beattie and Culver (1979) for four *Viola* species.

The dates on which measurements of flowering density were made also affect the calculated values of Ne (but not those of A, whose calculation does not include measurements of flowering density). *R. bulbosus* tends to flower synchronously. The number of plants in flower increases through the early part of the flowering season to reach a peak and then drops off rapidly. In 1987, most plants flowered within a 2-week period. Values of Ne calculated using flowering densities measured early in the flowering season were much lower than those calculated using measurements made later in the flowering season. This modifies

the conclusions drawn about levels of genetic drift in sub-populations. It is important, therefore, to use measurements which cover all of the flowering season. It would have been more accurate to include measurements of pollen flow made on several days throughout the flowering season, since the behaviour of pollinators is effected by flowering plant density. This was not possible due to a lack of time and to the weather.

This investigation highlights the problems associated with accurately estimating levels of gene flow. The measurements obtained are primarily applicable only to the time at which they were made. Conditions and population parameters are likely to vary within the study population from year to year, and even from day to day and site to site. Vegetative density of *R. bulbosus* was shown to exhibit significant variation between the two years in which measurements were made. It is likely that flowering density will show similar variation. It certainly showed significant variation between the two days on which measurements were made! Weather conditions, which vary from day to day and year to year, are known to markedly affect pollinator behaviour. For example, honey bees do not fly on cold days. In a summer when the frequency of cold days is high, pollen flow may be considerably reduced.

Limited gene flow may not always be detrimental to a population. It can be important in determining the ability of a group of plants occupying a particular environment to adapt to

that environment. If levels of gene flow are high, adaptation is less likely. The constant influx of genes from plants occupying different environments will cause dilution of the gene pool of the group of plants occupying the environment. Equally, where similar selective forces are acting throughout a population, local differentiation due to restricted gene flow will be reduced (Lynch, 1986). Where selection pressures are very high, differentiation can still occur over very short distances even in the presence of gene flow (Allard *et al*, 1972; Antonovics, 1971; Jain and Bradshaw, 1966). Similarly, with very restricted gene flow and weak selective pressures, local differentiation can occur due to random drift. Differentiation due to random drift will be more important where neighbourhood size is small compared with the area over which uniform selection pressures operate (Cahalan and Gliddon, 1985). The pattern of genetic variability within the overall population will be determined by the interaction of selective forces and gene flow (Antonovics, 1971; Felsenstein, 1979; Jain and Bradshaw, 1966; Levin and Kerster, 1968; Rai and Jain, 1982; Slatkin, 1985 a).

The population of *R. bulbosus* under study occupies several different habitats within the field at Rainbow Wood Meadow. In these, there is variation in soil moisture, shade, trampling pressure, fertility and competitive pressures. These would, in themselves, give rise to genetic variation in the population. This, coupled with the moderately restricted gene flow indicated by the results, would result in local differentiation of sub-

populations. This local differentiation may allow *R. bulbosus* to occupy more niches within the field than would otherwise be possible, thus increasing its overall success in terms of the numbers of surviving plants and the range of habitats it can occupy.

Felsenstein (1976) and Levin and Kerster (1974) suggest that, far from decreasing genetic variability, restricted gene flow may increase genetic variability within the overall population. Different sub-populations will contain different combinations of genes. So, whilst variability within each sub-population may be reduced, overall it will increase. Advantageous genes may also be fixed in the sub-populations. These may be spread throughout the population by rarer long distance gene flow. This, of course, may also apply to deleterious genes.

There are some limitations to the way in which gene flow was measured here. Two factors in the experimental method suggest that the values of Ne and A calculated here may be under-estimates.

Long distance pollen flow was not taken into account. Butterflies, which effect longer distance dispersal than bees (Herrera, 1987 a and b; Schmitt, 1980), were not included in estimates of dispersal distance. Also, bees which flew over 15 m were considered to have finished a foraging bout. Such long distance dispersal may have considerably altered the estimates obtained for Ne and A.

Long distance seed dispersal was not measured. This would have been very difficult without using some sort of marker on the seeds. Slatkin (1985 a) suggests that seed dispersal has much greater potential for gene flow than pollen dispersal. He reaches this conclusion because of the way in which plants colonize vacant habitats. Certainly, personal observations of the way in which *R. bulbosus* seeds stick to clothing and are carried by mud on shoes, suggests that long distance seed dispersal is a factor that should be taken into consideration.

In contrast to the discussion immediately above, two factors in the experimental method might lead to the conclusion that estimates of Ne and A given here are too large. In measuring pollen flow, it was assumed that bees travel in straight lines. Several authors have shown that this may not be so (Pleasants and Zimmerman, 1979; Pyke, 1978 b; Zimmerman, 1982 a). However, Levin *et al* (1971), who analysed consecutive flights for 2660 bees, showed linearity of flight direction. This did look to be the case here. However, the assumption may have resulted in a slight over-estimation of pollen dispersal distances.

The effect of the presence of other species in the field which was discussed earlier may also reduce values of Ne and A. The importance of this phenomenon will be affected by two main factors: the flowering density of *R. bulbosus*, and the presence or absence of other species that are flowering. Flowering densities of *R. bulbosus* are low early and late in its flowering season, but

early in the season few other species are in flower, reducing competition for pollinators. Pollen flow may be increased in this situation, since pollinators will have to fly long distances between flowers. Later in its flowering season *R. bulbosus* is one of many other species which are in flower, including the two other buttercup species present in the field. Pollen flow is likely to be decreased by the presence of other flowering plants at this time. Pollinators may be particularly unlikely to distinguish between flowers belonging to the three species of buttercup.

The estimates of Ne and A calculated here only give a measure of potential gene flow at one point in time. To measure actual gene flow would have involved following a genetic marker through the population. As no morphological markers could be found, this would have meant using an electrophoretic marker. *R. bulbosus* is not a common subject for electrophoretic techniques. It was decided, after a pilot investigation, that the time and effort that would be involved in developing an adequate electrophoretic assay would be better utilised elsewhere. Therefore, it was decided to look only at potential gene flow. In doing this, much thought was given to methods which would increase the accuracy of these estimates over those obtained in previous studies. For instance, the use of estimates of pollen carryover. Some inaccuracies do still exist in the methods, but these have been taken into account when discussing the results obtained.

CHAPTER 5

GENERAL DISCUSSION

The two aspects of the research undertaken (sexual allocation and gene flow) have been discussed extensively in Chapters 3 and 4. The aim of this chapter is not, therefore, to repeat these discussions but to summarise the results and discuss whether the two areas of research have any implications with respect to each other. Chapter 3 was concerned with sexual allocation strategy in *R. bulbosus*. The strategy adopted in the design of the experiments was to invest a lot of time on a small sample of *R. bulbosus* plants, rather than trying to study a whole population. In all, only 15 different clone types were used. In adopting this strategy, more measurements could be made on each plant, and the variability within the sample could be reduced. This meant that a clearer idea of the cause of variability in sexual allocation strategy could be obtained. Since the overall sample size was so small, these results should not be extrapolated to make broad conclusions concerning the population of *R. bulbosus* as a whole. Certain conclusions can be drawn about the study population. Experiment 3.2.2.b) showed that, generally, the plants were adopting similar strategies under similar conditions in the way in which they allocate resources to female function

through the flowering season. That is, there was a relative increase in investment in the female function, although a decrease in total allocation to reproduction (for one plant, relative investment remained constant). Seven of the clones used in experiment 3.2.2.c) also exhibited this strategy. The other three clones maintained the same relative gender allocation through time, but allocated less to reproduction overall.

Data obtained by Stephenson (1984) on *Lotus corniculatus* supported the hypothesis that production of surplus flowers will be selected for the male contribution to fitness. If this were the case in this study, it would be expected that later flowers would be relatively more male. This is not the case. A possible explanation is as follows:

Later in the season, the plant will be maturing seeds produced earlier in the season. Any further production of pollen and ovules would detract from the resources which can be allocated to this function. So, there is a decrease in allocation to production of new gametes. The fitness gained through pollen production will decrease through the flowering season, as there are fewer ovules of conspecifics to be fertilized. Those which are fertilized later in the season may not be matured by the plant if it has already matured a number of seeds produced earlier in the season. If the seeds produced earlier in the season were lost, however, the benefit of continued investment in ovule production would outweigh the cost. It is likely that these

ovules would be fertilized, since there would be some pollen production by conspecifics and less competition with ovules of conspecifics. The plant would, therefore, produce some seed, rather than none at all. So, the plant would maintain its ovule production at a higher level relative to pollen production.

Experiment 3.2.2.d) showed some evidence for ESD in the population of *R. bulbosus* under study.

Chapter 4 was concerned with gene flow in the population of *R. bulbosus* at Rainbow Wood Meadow. This study was designed to make general predictions about the population as a whole in terms of levels of gene flow. The calculations of neighbourhood size and area suggest that gene flow in this population is restricted.

In Fisher's (1930, quoted from Charnov, 1982) basic model of sexual allocation, a 1:1 allocation ratio is predicted under random mating. The results presented in Chapter 4 indicate that gene flow in the population of *R. bulbosus* at Rainbow Wood Meadow is moderately restricted, because of localized pollen and seed dispersal. A major implication of this is that mating is non-random. Consequently, a 1:1 allocation ratio is not necessarily to be expected.

One consequence of localized pollen dispersal may be local mate competition (LMC) (Charnov, 1982; Lloyd, 1984 b). The theory of LMC was developed by Hamilton (1967) and was discussed in the

introduction to Chapter 3. Briefly, local pollen dispersal will restrict the number of mates available to a plant and will result in its pollen competing for these mates (Lloyd, 1984 b). The male gain curve will show decreasing returns for increased production of pollen due to the increasingly detrimental effects of pollen competition. Increased allocation to the female function, or decreased allocation to the male function will, therefore, be selected for. This pressure will be enhanced in a subdivided population due to inbreeding (resulting from restricted gene flow) within subpopulations. Charlesworth and Charlesworth (1981) show that self-fertilization will have similar effects to LMC. Although *R. bulbosus* has been shown to be self-sterile, the effects of inbreeding will result in some mates being very closely related.

This research has indicated that seed as well as pollen dispersal is localised in *R. bulbosus* (Chapter 4). Lloyd (1984 b) predicts that localised seed dispersal will result in local resource competition (LRC). This will be manifested as sib competition. If the seed shadows of the various mates of a pollen donor do not overlap, an increase in the male function of that donor will be selected for, as LRC among its (paternally derived) progeny will be absent. However, it is probable that in *R. bulbosus* the seed shadows do overlap to some extent. Lloyd (1984 b) predicts that the effects of LMC and LRC will cancel each other out in natural populations where seed shadows do not overlap. Where there is some overlap, the effects may not

completely cancel each other, but there is likely to be some reduction in the effects of both.

Bulmer and Taylor (1980) show that the sex ratio will be biased towards the male or female function depending upon which disperses more widely or more evenly, pollen or seed. In addition, there will be some bias towards the female function (seed production) because dispersal occurs after fertilization. The experiments performed here suggest that both pollen and seed dispersal are restricted, although the mean dispersal distance for pollen (115.55 cm) was higher than that for seeds (27.54 cm).

So, for the population of *R. bulbosus* under investigation, the effects of LRC and LMC would, overall, be expected to result in male function being favoured. The theory discussed by Bulmer and Taylor (1930) would, with respect to *R. bulbosus*, back up this prediction, since pollen disperses more widely than seed.

Because anther counts, rather than pollen counts, were used in the original survey of sexual allocation ratio at Rainbow Wood, the bias of this population cannot be commented upon. However, looking at measurements of $\underline{G}$ in experiment 3.2.2.c), where there was no effect of treatment, it can be seen that some clones are overall female biased ($G > 0.5$), some male biased ($G < 0.5$), and some show no bias ($G = 0.5$). Overall, then, it would appear that the population of plants used in that experiment showed an allocation ratio of close to 1:1. This was an experimental

population of plants collected from various other populations whose genetic structure was not known. The sample size was also very small. It would be improper, therefore, to use this population as anything other than a suggestion of what might be happening in *R. bulbosus*.

APPENDIX 1

DEFINITION OF TERMS

1) ESS - evolutionarily stable strategy. The equilibrium value of a trait (Maynard-Smith, 1976, quoted in Charnov, 1982)

2) ESD - environmental sex determination

3) RS - reproductive success

4) G_i - functional femaleness (Lloyd, 1979)

5) A_i - functional maleness (Lloyd, 1979)

6) Ne - effective population size (estimated here by neighbourhood size) (Wright, 1943)

7) A - neighbourhood area (Wright, 1943)

8) ANOVA - analysis of variance

9) ANCOVA - analysis of co-variance

10) LMC - local mate competition (Lloyd, 1984 b)

11) LRC - local resource competition (Lloyd, 1984 b).

12) P/O - pollen: ovule ratio

13) vP/O - viable pollen: total ovule ratio

14) tratio - ratio of total pollen: total ovule

15) vratio - ratio of viable pollen: 'effective' ovules

16) vO - 'effective' ovules

17) tpoll - total pollen count

18) vpoll - viable pollen count

19) M - $\frac{\sum_{i=1}^{n} \text{pollen count per flower for flowers 1 to n}}{\sum_{i=1}^{n} \text{ovule count per flower for flowers 1 to n}}$

20) vM - $\frac{\sum_{i=1}^{n} \text{viable pollen count per flower for flowers 1 to n}}{\sum_{i=1}^{n} \text{'effective' ovule count per flower for flowers 1 to n}}$

21) Tf - total allocation to gametes per flower

22) Tp - total allocation to gametes per plant

23) vTf - total allocation to viable gametes per flower

24) vTp - total allocation to viable gametes per plant

25) g_i - gynoecial production (Lloyd, 1979)

26) a_i - androecial production (Lloyd, 1979)

27) E - equivalence factor relating pollen production to ovule production

28) MSD - minimum significant difference

29) w/w - weight for weight

30) v/v - volume for volume

31) BA - Benzyladenine

32) PAR - Photosynthetically Active Radiation

33) psi - pounds per square inch

34) SCM - Sham Castle Meadow

35) FR - Firing Range

36) RB - Rock Border

37) n_1 - number of males in population

38) K_2 - number of pollen grains produced by each male

39) n_2 - number of females in population

40) K_1 - number of seeds produced by each female

41) n_3 - number of hermaphrodites in population

42) $f.K_1$ - number of seeds produced by each hermaphrodite

43) $m.K_2$ - number of pollen grains produced by each hermaphrodite

44) m - proportion of hermaphrodites' fitness transmitted through male function

45) f - proportion of hermaphrodites' finess transmitted through female function

46) Wf - fitness of females

47) Wm - fitness of males

48) Wn - fitness of hermaphrodites

49) K - number of offspring produced by the population

50) r - allocation to male function

51) n - size of mating group

APPENDIX 2

RESULTS OF EXPERIMENT TO DETERMINE FLOWERING DENSITY AND SEQUENCE WITHIN A 10 M^2 PERMANENT PLOT AT THE FIELD SITE

Large Quad. No.	Small Quad. No.	Plant Co-Ord.		No. of Flwrs. on Date of Visit				
		Bottom	Side	28/4	12/5	19/5	26/5	9/6
1	1	15.5	11.0	0	0	0	0	0
		5.5	8.5	0	1	1	S	S
		3.5	22.0	0	1	1	S	S
		16.0	20.0	0	1	3	S	S
		7.0	25.0	0	1	8	1	S
		19.0	24.0	0	1	6	2	S
1	2	7.0	10.5	0	1	4	S	S
		7.5	2.0	0	1	2	S	Sg
		5.5.	1.0	0	1	1	S	Sg
		9.5	12.5	0	1	3	S	S
		11.5	8.5	0	1	5	2	1(S)
		16.5	5.0	0	1	1	S	S
		21.5	11.5	0	0	0	1	S
		13.0	16.0	0	0	1	5	S
		21.5	11.5	0	0	0	0	0
1	3	1.0	22.0	0	1	9	1	1(S)
		8.0	5.5	0	1	1	S	S
		8.5	8.5	0	1	1	S	Sg
		8.0	20.0	0	0	4	S	S
		18.0	24.0	0	1	2	10	3(S)
1	4	5.5	16.0	0	1	1	1	S
		4.5	20.5	0	1	6	1	S
		12.5	14.5	0	1	17	S	S
		18.5	6.5	0	0	0	2	S
		22.0	13.5	0	1	5	1	S
		21.0	15.5	0	1	7	1	S
		3.0	2.5	0	0	0	0	0

Large Quad. No.	Small Quad. No.	Plant Co-ord.		No. of Flwrs. on Date of Visit				
		Bottom	Side	28/4	12/5	19/5	26/5	9/6
2	1	13.5	3.5	0	0	0	0	0
		16.5	4.5	0	0	0	0	0
2	2	3.5	5.5	0	0	0	0	0
		4.5	17.5	0	0	1	S	Sg
		13.5	7.5	0	0	0	0	0
3	2	12.0	7.0	1	0	0	0	0
		19.0	4.0	0	0	0	0	0
3	4	5.5	11.0	0	0	0	0	0
		11.5	21.5	0	1	1	S	Sg
3	1	8.5	4.5	0	0	1	S	S
		4.0	12.5	0	0	0	0	0
		1.5	14.5	0	0	0	0	0
		11.5	18.5	0	0	0	0	0
		25.0	15.5	0	0	0	0	0
		22.5	25.0	0	0	0	0	0
3	2	9.5	2.5	0	0	1	S	Sg
		25.0	16.0	0	1	3	S	S
		20.0	0.0	0	0	0	0	0
3	3	14.5	3.0	0	0	0	0	0
		21.0	0.0	0	0	0	0	0
3	4	6.0	7.0	0	0	0	0	0
		4.5	9.5	0	0	0	0	0
		4.5	12.5	0	0	0	0	0
		9.0	9.0	0	0	0	0	0
		15.0	8.5	0	0	0	0	0
		14.5	13.5	0	0	0	0	0
		20.5	25.0	0	0	0	0	0
		22.0	8.5	0	1	1	S	S
4	1	7.0	8.5	0	1	4	1	S
		6.0	21.0	0	0	0	0	0
		13.0	16.0	0	0	0	0	0

Large Quad. No.	Small Quad. No.	Plant Co-ord.		No. of Flwrs. on Date of Visit				
		Bottom	Side	28/4	12/5	19/5	26/5	9/6
4	4	3.5	15.0	0	0	0	0	0
		21.0	10.0	0	1	2	S	S
5	1	3.5	3.5	1	0	0	0	0
		5.0	15.0	0	0	0	0	0
		2.5	21.5	0	0	0	0	0
		4.5	23.0	0	0	0	0	0
		7.0	25.0	0	0	1	S	Sg
		10.0	3.0	0	0	1	S	Sg
		14.0	19.0	0	0	0	0	0
		17.0	20.0	0	0	0	0	0
		21.0	8.0	0	0	0	0	0
5	2	5.5	11.0	0	1	1	S	Sg
		6.5	10.0	0	0	0	0	0
		12.5	10.0	0	1	0	0	0
		14.0	7.5	0	0	0	0	0
		14.0	9.5	0	0	0	0	0
		13.5	20.0	0	0	0	0	0
		21.5	2.0	0	1	1	S	Sg
5	3	18.5	13.0	0	1	2	S	Sg
5	4	7.5	0.0	0	0	0	0	0
6	1	5.0	21.0	0	0	1	S	Sg
		17.5	19.0	0	0	1	S	Sg
		21.0	10.5	0	0	1	S	Sg
		23.0	8.5	0	0	0	0	0
		20.5	3.0	0	0	0	0	0
		14.0	6.0	0	1	1	S	Sg
6	2	4.0	8.5	0	0	0	0	0
		9.0	4.5	0	0	0	0	0
		3.0	22.0	0	0	0	0	0
		6.5	21.0	0	0	0	0	0
		11.0	24.0	0	1	3	S	Sg
		15.5	20.5	0	1	2	S	Sg

Large Quad. No.	Small Quad. No.	Plant Co-ord.		No. of Flwrs. on Date of Visit				
		Bottom	Side	28/4	12/5	19/5	26/5	9/6
6	2	18.0	15.0	0	1	2	S	Sg
		24.0	23.0	0	0	0	0	0
6	3	3.0	21.0	0	1	2	S	S
		9.0	21.5	0	1	2	2	S
		9.5	17.0	0	0	0	0	0
		9.0	12.5	0	0	0	0	0
		14.0	3.0	0	0	0	0	0
		23.5	3.5	0	0	0	0	0
6	4	5.5	21.5	0	0	0	0	0
		5.0	25.0	0	1	2	S	Sg
		12.5	17.0	0	0	0	0	0
		17.0	17.0	0	1	1	S	Sg
		20.5	15.5	0	1	3	S	Sg
		23.0	2.0	0	1	4	S	S
7	1	9.5	18.5	0	0	0	0	0
		11.0	18.5	0	0	0	0	0
		19.5	4.5	0	1	*3	Sg	Sg
		23.0	15.0	0	0	0	0	0
		23.5	21.0	0	1	5	S	S
		25.0	20.0	0	0	0	0	0
7	2	9.0	7.5	0	1	*1	0	0
		6.5	15.0	0	0	0	0	0
		5.5	17.0	0	1	*2	Sg	Sg
		18.5	5.5	0	1	1	S	Sg
7	3	3.0	5.5	0	1	*1	Sg	Sg
		5.5	20.5	0	1	1	S	Sg
		20.5	21.0	0	1	1	0	0
7	4	6.0	10.0	0	0	0	0	0
		11.0	18.5	0	0	0	0	0
		16.0	2.0	0	1	*4(2)	S	Sg
8	1	6.0	7.5	0	1	1	S	Sg
		19.0	4.0	0	0	0	0	0

Large Quad. No.	Small Quad. No.	Plant Co-ord.		No. of Flwrs. on Date of Visit				
		Bottom	Side	28/4	12/5	19/5	26/5	9/6
8	2	4.5	9.5	0	1	3	S	S
		9.5	14.5	0	0	0	0	0
		10.5	12.5	0	1	2	1	S
		24.0	15.5	0	1	1	1	S
8	3	8.0	20.0	0	1	3	S	S
		12.0	9.0	0	0	0	0	0
		2.0	25.0	0	0	0	0	0
8	4	18.0	16.0	0	0	0	0	0
		20.5	12.0	0	0	0	0	0
9	1	6.0	1.0	0	1	2	S	S
		9.5	6.5	0	0	0	0	0
		11.5	6.5	0	0	1	S	Sg
		15.0	6.5	0	0	0	0	0
		3.0	21.0	0	0	0	0	0
		17.0	21.0	0	0	0	0	0
		15.0	12.0	0	1	3	S	Sg
		23.0	13.0	0	1	2	S	Sg
9	2	6.0	5.0	0	0	0	0	0
		18.0	25.0	0	1	7	S	S
		20.0	9.0	0	1	1	S	S
		23.0	6.0	0	1	1	S	S
9	3	6.5	2.0	0	1	2	S	Sg
		4.0	13.0	0	1	4	S	Sg
		7.5	2.0	0	1	9	1	S
		11.5	18.5	0	0	3	S	Sg
		16.0	17.0	0	1	2	Sg	Sg
		15.0	13.0	0	1	1	S	S
		22.0	24.5	0	1	1	S	Sg
		13.0	24.0	0	0	0	0	0
		21.0	0.0	0	1	1	Sg	Sg

Large Quad. No.	Small Quad. No.	Plant Co-ord.		No. of Flwrs. on Date of Visit				
		Bottom	Side	28/4	12/5	19/5	26/5	9/6
9	4	8.5	3.5	0	1	1	S	S
		3.0	10.5	0	0	2	S	Sg
		8.0	17.0	0	0	0	0	0
		25.0	20.0	0	1	4	S	Sg
		25.0	4.0	0	1	3	Sg	Sg
		17.0	2.0	0	1	4	S	S
10	1	8.5	3.5	0	1	*1	0	0
		10.0	6.0	0	0	0	0	0
		6.0	11.0	0	0	D	E A	D
		9.0	9.0	0	0	0	0	0
		16.0	6.0	0	0	0	0	0
10	2	1.5	6.0	0	0	0	0	0
		1.5	8.0	0	0	0	0	0
		1.5	15.0	0	0	0	0	0
		16.0	3.0	0	0	0	0	0
		18.0	7.0	0	0	0	0	0
		18.0	9.0	0	0	0	0	0
		16.0	7.5	0	0	0	0	0
10	3	0.0	11.0	0	0	0	0	0
		3.0	7.0	0	0	0	0	0
		12.0	21.0	0	0	0	0	0
		18.0	12.5	0	0	0	0	0
		3.0	11.0	0	0	0	0	0
		6.0	15.0	0	0	0	0	0
		7.0	15.0	0	0	0	0	0
		14.0	19.0	0	0	0	0	0
		23.5	25.0	0	0	0	0	0

KEY:

* = Flower eaten

S = Seeds

Sg = Seeds gone

APPENDIX 3

CALCULATION OF THE CONSTANT "K" TO CORRECT FOR THE LEPTOKURTOTIC DATA USED IN CALCULATIONS OF Ne AND A

1. Estimation of levels of kurtosis

a) For pollen (not taking into account carryover):

$\gamma_2 = 8.17$

b) For pollen (taking into account pollen carryover):

$\gamma_2 = 8.89$

c) For overall seed dispersal distances:

$\gamma_2 = 0.50$*

* Since seed dispersal was not significantly kurtosed, this calculation was taken no further.

2. Calculation of α (from Crawford, 1984 a)

$$\log_{10} \alpha = 0.341 \log_{10}\gamma_2 - 0.156 \text{ for } 1 < \gamma_2 < 15$$

$$\log_{10} \alpha = 0.295 \log_{10}\gamma_2 - 0.097 \text{ for } 15 < \gamma_2 < 50$$

a) For pollen (not taking into account carryover):

$$\log_{10} \alpha = 0.341 \log_{10} 8.17 - 0.156$$

$$\alpha = \underline{1.429}$$

b) For pollen (taking into account carryover):

$$\log_{10} \alpha = 0.341 \log_{10} 8.89 - 0.156$$

$$\alpha = \underline{1.471}$$

3. Calculation of A_1 (corrected neighbourhood area for pollen only)

$$A_1 = 2^{2\alpha} [\Gamma(2\alpha+1)\ \Gamma(\alpha)/\Gamma(3\alpha)]\Pi\sigma^2$$

a) For pollen (not taking into account carryover):

$$A_1 = 2^{2 \cdot 858} [\Gamma(3.858)\ \Gamma(1.429)/\Gamma(4.287)]\Pi\ 7077.8$$

$$A_1 = \underline{82630.74}$$

b) For pollen (taking into account carryover):

$$A_1 = 2^{2 \cdot 942} \Gamma(3.942)\ \Gamma(1.471)/\Gamma(4.413)]\Pi\ 19335.89$$

$$A_1 = \underline{223635.93}$$

4. Calculation of A (uncorrected neighbourhood area for pollen only)

$$A = 4\Pi\sigma^2$$

a) For pollen (not taking into account carryover):

$$A = 4\Pi 7077.8$$

$$A = \underline{88942.26}$$

b) For pollen (taking into account carryover):

$$A = 4\Pi 19335.89$$

$$A = \underline{242981.95}$$

5. Calculation of k

$$A_1/A = k/4$$

a) For pollen (not taking into account carryover):

$$82630.74/88942.26 = k/4$$

$$Kp = \underline{3.716}$$

b) For pollen (taking into account carryover):

223635.93/242981.95 = k/4

Kp = <u>3.681</u>

N.B.: γ_2 is a measure of observed leptokursosis. It can be calculated using the equation given in Crawford (1984 a). In this case, the SAS statistical package was used to calculate observed leptokursosis of data.

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